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PROPERTIES OF TRANSAMINASES AND GLUTAMIC DEHYDROGENASE IN THE COCKROACH *PERIPLANETA AMERICANA*

J. W. McALLAN* and W. CHEFURKA

Department of Zoology, University of Western Ontario, and the Pesticide Research Institute, Canada Department of Agriculture, London, Ontario, Canada

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Abstract—1. Glutamate–aspartate transaminase was purified from homogenates of cockroaches and of pig heart.

2. The properties of the transaminases were compared with regard to substrate affinity, pH, equilibrium constant and temperature.

3. It was found that borate had a profound effect on the equilibrium constant which was a function of pH. An explanation is proposed in terms of a complex-formation between borate and the *cis*-diol structure of oxaloacetate at alkaline pH.

4. The transamination of alanine and certain other amino acids with α-ketoglutarate was studied chromatographically.

5. Glutamic dehydrogenase activities from the fat body of cockroaches and beef liver were compared; a difference in the substrate affinity and equilibrium constant was noted.

6. The relationship between the properties of the transaminases and glutamic dehydrogenase are discussed with regard to the high free amino acid content of the cockroach hemolymph.

INTRODUCTION

TRANSAMINATION in insects was demonstrated first by Barron & Tahmisian (1948) in the thoracic muscles of the cockroach, *Periplaneta americana*. More recently Bheemeswar & Sreenivasaya (1952), Shyamala & Bhat (1955) and Fukuda (1957) have studied the glutamate–aspartate and glutamate–alanine transaminases in *Bombyx mori*, and have suggested that transamination provides the building blocks for protein synthesis in insects.

An extensive survey of amino acid metabolism in *Schistocerca gregaria* was carried out by Kilby & Neville (1957), who demonstrated chromatographically the production of glutamate in reaction mixtures containing α-ketoglutarate, fat body homogenate and each of seventeen amino acids. They found also that glutamic dehydrogenase, arginase and the amino acid oxidases are present in the fat body of the locust.

Most of the work on insect transaminases has been of a qualitative nature, and no attempt has been made to date to characterize these enzymes in terms of accepted enzymological parameters. Knowledge of the effect of these parameters on enzymic activity would be of value to the comparative biochemist and physiologist. Such

* Present address: Research Station, Canada Department of Agriculture, Fredericton, New Brunswick, Canada.

knowledge is an essential prerequisite to further studies on the function of transamination in growth and differentiation. Therefore we undertook a study of some of the enzymes regulating amino acid metabolism in the cockroach, particularly glutamic dehydrogenase and glutamate–aspartate and glutamate–alanine transaminases, from this particular point of view.

MATERIALS AND METHODS

The insects were adult *Periplaneta americana* L. taken from a stock laboratory culture.

The chemicals used in this work were purchased commercially. Four buffers were prepared for the determination of the effect of pH on glutamate–aspartate transaminase activity: (1) Clark and Lubs' buffer consisting of 0·2 M boric acid and 0·2 M KCl, titrated with 0·2 M NaOH for pH values more alkaline than 8·0; (2) McIlvaine's buffer containing 0·2 M Na_2HPO_4 and 0·1 M citric acid for pH values more acid than 8·0; (3) Tris buffer containing 0·1 M Tris(2-amino-2-hydroxymethyl-1, 3-propanediol) and an equal volume of 0·1 M NaH_2PO_4 titrated with 0·1 M NaOH; and (4) phophate buffer containing 0·25 M NaH_2PO_4 titrated with 0·1 M NaOH. All solutions of amino and keto acid substrates were neutralized and their pH adjusted with NaOH.

Protein concentrations were determined by the method of Lowry *et al.* (1951) using Folin's phenol reagent and crystalline serum albumin as standard. Measurements of optical densities were made with a Beckman spectrophotometer, model DU, at 500 mμ.

Glutamate–asparate transaminase activity was assayed spectrophotometrically according to Green *et al.* (1945). In reaction (i)

$$\underset{(1)}{\text{aspartate}} + \underset{(2)}{\alpha\text{-ketoglutarate}} \underset{\text{B}}{\overset{\text{A}}{\rightleftharpoons}} \underset{(3)}{\text{glutamate}} + \underset{(4)}{\text{oxaloacetate}} \qquad \text{(i)}$$

the accumulation or disappearance of oxaloacetate is followed at 280 mμ. The millimolar extinction coefficient (K_e) of the reaction was calculated according to Cohen (1955) from the formula

$$K_e = \frac{1}{K_3 + K_4 - K_1 - K_2}.$$

For the forward reaction (A) K_e was 2·9 and for the reverse reaction (B) it was 8·4. The difference in these values for the forward and reverse reactions was due to the fact that Beer's law was obeyed only for low concentrations of oxaloacetate. No significant difference in values for the millimolar extinction coefficients of the reactants and products was observed using the four buffers above, except in concentrated solutions of oxaloacetate. For concentrations of and above 2 mM oxaloacetate in borate buffer, the optical densities were slightly larger than those recorded for solutions of oxaloacetate in the other three buffers.

A unit of enzymic activity was defined as that amount of enzyme producing 0·588 mg of oxaloacete in 10 min at 22°C in the standard reaction mixture. This

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unit is equivalent to that defined by Green *et al.* (1945), which produced 100 μl of CO_2 in a manometric assay. Where only a comparison of enzymic activities was required the activity was expressed as change in optical density per 10 min in the standard reaction. The standard reaction (i,A) contained 0·5 ml of 0·1 M aspartate, 0·5 ml of 0·1 M α-ketoglutarate, 0·1 ml of enzyme solution and 3·9 ml of buffer, and the reverse reaction (i,B) contained glutamate and oxaloacetate as reactants.

Glutamate–alanine transaminase was assayed according to Green *et al.* (1945). In the reaction:

$$\text{alanine} + \alpha\text{-ketoglutaric acid} \rightleftharpoons \text{glutamic acid} + \text{pyruvic acid} \qquad \text{(ii)}$$

the pyruvate was reacted with 2 per cent salicylaldehyde and measured at 440 mμ. Specifically, 1 ml of a solution containing less than 6 μM pyruvate was mixed with 1 ml of 62·5 per cent KOH and 0·5 ml of 2 per cent salicylaldehyde in ethanol. After 10 min 22·5 ml of H_2O were added and the optical density measured at 440 mμ. The concentrations of unknown samples were determined from a calibration curve.

Paper chromatography of amino acids was performed by the ascending method using 80 per cent phenol or butanol–acetic acid–water (4 : 1 : 5) as solvents. About 30 μl of the test solution was spotted on Whatman No. 1 filter paper. The developed chromatograms were dipped in 0·25 per cent ninhydrin in acetone. Maximum color development was achieved by storing the chromatograms in the dark for 18 hr as described by Block *et al.* (1952). The test mixture consisted of 0·1 M α-ketoglutarate and the appropriate amino acids at a final concentration of 0·1 M. These were incubated with 0·2 ml of a dialysed roach homogenate for 1 hr at 38°C at pH 7·8 in McIlvaine's buffer.

Glutamic dehydrogenase activity was measured according to Strecker (1955). The reaction mixture consisted of 0·3 ml of 0·1 M glutamate, 0·2 ml of 3 mM diphosphopyridine nucleotide (DPN), 0·1 ml of homogenate and 2·4 ml of buffer to a total volume of 3·0 ml. Controls contained boiled enzyme. The activity was assayed using a Beckman model DU spectrophotometer at 340 mμ, by noting the increase in optical density due to the formation of reduced diphosphopyridine nucleotide (DPNH). The reaction in the reverse direction consisted of 0·3 ml of 0·1 M α-ketoglutarate, 0·3 ml of 0·1 M ammonium chloride, 0·3 ml of 0·45 mM DPNH, 0·3 ml of extract of roach fat body and 1·8 ml of buffer. In control tests ammonium chloride was replaced by buffer.

It is conceivable that the activity of the DPNH oxidase system could counteract the formation of DPNH or contribute to its disappearance in the assays of glutamic dehydrogenase. The possibility of this interference was checked in the presence of $1{\cdot}3 \times 10^{-3}$ M cyanide which is a potent inhibitor of the DPNH oxidase system. Neither the rate of formation of DPNH nor its disappearance was altered, suggesting that the DPNH oxidase system was very low under our conditions. This was confirmed in the controls of glutamic dehydrogenase assays which contained all the components, including DPNH, but not ammonium chloride. Under these conditions the activity of the DPNH oxidase system was occasionally of the order

of 0·003 optical density units in 10 min or approximately 8 per cent of the total activity. In many preparations no activity of DPNH oxidase system could be detected.

The endogenous amino acids were removed from the extracts either by dialysis against water for 24 hr at 0·5°C or by precipitation of the protein enzyme with excess ammonium sulphate. The precipitate was then resuspended in water. As a result of either procedure, the activity of the extracts was greatly enhanced.

Purification of the glutamate–aspartate transaminase

Glutamate–aspartate transaminase was purified from pig heart and whole roaches following the procedure outlined by Green *et al.* (1945). Tissues were ground in a Waring blendor in four times their weight of water and then filtered through several layers of gauze. The residue was washed with water and the washings added to the filtrate which was then centrifuged at 15,000 *g* for 15 min. This filtrate was designated as the "first supernatant". The residue was discarded and 30 g of solid ammonium sulphate per 100 ml of supernatant were added. The resulting precipitate was removed by centrifugation at 15,000 *g* for 10 min and the supernatant was designated as the "second supernatant". To it were added 10 g of ammonium sulphate per 100 ml. Most of the enzymic activity resided in the residue which was named the "first residue" which was obtained by centrifugation at 15,000 *g* for 10 min. After resuspending this residue in water, 46 g of solid ammonium sulphate per 100 ml were added. The precipitate was removed by centrifugation. Since most of the activity resided in the precipitate the supernatant was discarded. The "second residue" was resuspended in water, brought quickly to 60°C and then cooled immediately. The coagulated precipitate was removed by centrifugation and the supernatant was used as the source of enzyme. It was put into several small vials and stored in a deep-freeze.

The activity of purified glutamate–aspartate enzyme from both pig heart and roaches was not affected by additions of the coenzyme, pyridoxal phosphate. Hence the above preparations will be referred to as "unresolved" enzyme. However, a modification of this procedure did resolve the enzyme into apoenzyme and coenzyme. In the modified procedure, the fraction termed the "second residue" was suspended in water and 60 g of ammonium sulphate per 100 ml added. The resulting precipitate was collected by centrifugation, resuspended in 20 per cent ammonium sulphate, and dialysed against 20 per cent ammonium sulphate for 24 hr at 0·5°C. The dialysed solution possessed no transaminase activity, but when 3 ml of dialysed solution and 1 ml of pyridoxal-5-phosphate (20 μg/ml) were mixed and incubated for 20 min, glutamate–aspartate enzymic activity was restored to the level of activity of the "second residue".

RESULTS

1. *Glutamate–aspartate transaminase*

(a) *Purification.* The results of the purification of glutamate–aspartate transaminase from pig heart and roaches are compared in Table 1. It is apparent that

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roach enzyme was purified about eightfold and pig heart enzyme fivefold. It is not possible to assess differences in the degree of purification, but it should be noted that the enzyme from both sources was purified by the same procedure, that initial enzymic activities per milligram of tissue are of the same order, and that both resolved enzymes are activated by the same coenzyme, pyridoxal-5-phosphate.

TABLE 1—PURIFICATION OF GLUTAMATE–ASPARTATE TRANSAMINASES FROM ROACHES AND PIG HEART

Fraction	Volume (ml)	Units per ml	Total units	Specific activity*	% Recovery
Whole roaches					
Filtered extract	190	11·0	2090	0·9	
1st supernatant	190	10·5	1995	1·8	95
2nd supernatant	150	10·0	1500	6·4	71
1st residue	150	7·0	1050	4·6	50
2nd residue	25	14·4	360	2·4	17
Supernatant	22	6·0	132	7·5	6
Pig heart					
Filtered extract	2200	15·0	33,000	5·3	
1st supernatant	1891	10·5	19,860	5·3	60
2nd supernatant	1630	10·0	16,300	12·5	50
1st residue	400	14·0	5600	1·4	17
2nd residue	86	75·0	6000	9·0	18
Supernatant	80	50·0	4000	24·0	12

* Specific activity is the units of enzymic activity per milligram of protein.

(b) *Effect of enzyme concentration on reaction rate.* Initial experiments showed that the rate of the transaminase reaction was approximately linear for the first 15 min when substrate concentrations were 0·01 M, therefore the rate of reaction is expressed in optical density per 10 min. The data in Fig. 1 show that the activity of purified roach enzyme was a linear function up to 80 μg of protein/ml. Above this concentration of protein the substrates' concentration may become limiting, causing an inflection in the plot. In all subsequent studies with purified enzyme the protein concentration was 80 μg/ml.

(c) *The effect of substrates' concentration on glutamate–aspartate enzymic activity.* Nisonoff & Barnes (1952) and Cook (1957) have studied Michaelis' constants of this enzyme, and it is evident from the nature of the reaction that two such constants are involved. However, for the purpose of comparing the enzyme from pig heart and roaches it was considered feasible to vary the concentrations of both substrates equally, and to compare the results graphically. Since the extent of enzyme-substrate interaction is dependent on the amount of enzyme present, roach and pig heart enzyme solutions were adjusted first to the same activity at a fixed concentration of substrates.

The results of these tests are shown in Fig. 2. Equal amounts of enzymic activity from pig heart and roaches appear to have approximately the same affinity for substrates.

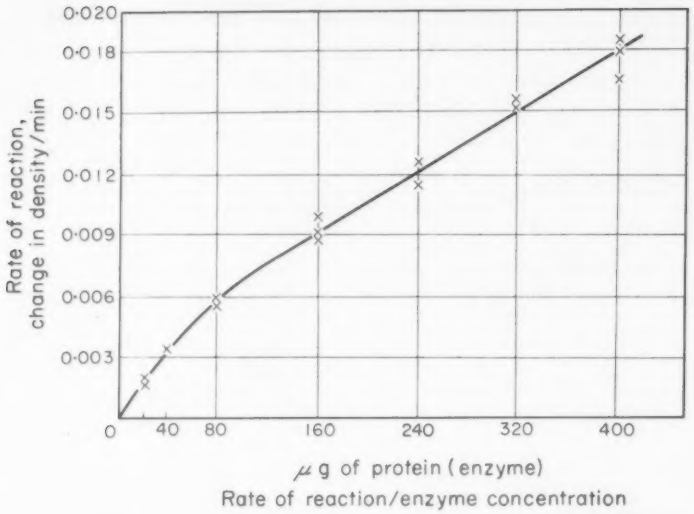

FIG. 1. The effect of concentrations of roach glutamate–aspartate enzyme on initial rates of reaction, using the spectrophotometric method of Green *et al.* (1945), Clark and Lubs' buffer, pH 8·3. The reaction consisted of 0·5 ml of 0·1 M aspartate, 0·5 ml of 0·1 M α-ketoglutarate, 0·1 ml of enzyme at several concentrations and 3·9 ml of buffer. Temperature 22°C.

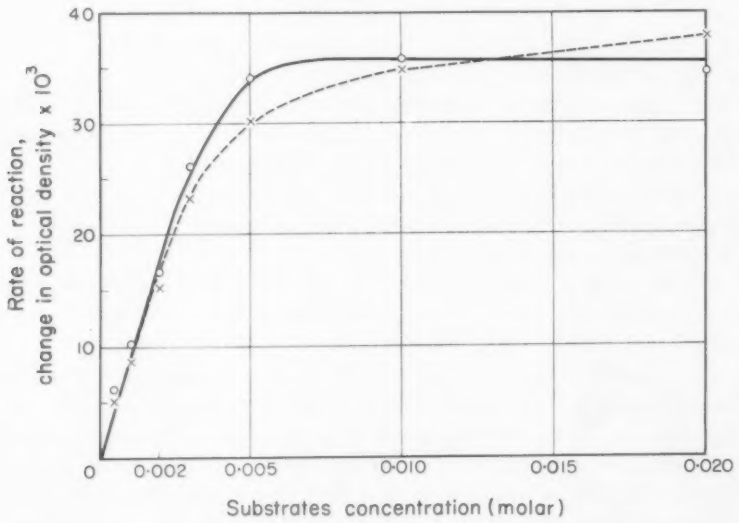

FIG. 2. Comparison of the affinity for substrates of glutamate–aspartate transaminase from roaches (O——O) and pig heart (X- - - -X). The reaction contained 0·1 ml of enzyme, 3·9 ml of borate buffer, pH 8·3, and 0·5 ml of equimolar concentrations of aspartate and α-ketoglutarate.

(d) *The effect of pH and buffers on glutamate–aspartate transaminase.* The effect of pH on the initial rate of reaction of purified enzyme from both sources was first studied using McIlvaine's buffer at pH values less than 8·0 and Clark and Lubs'

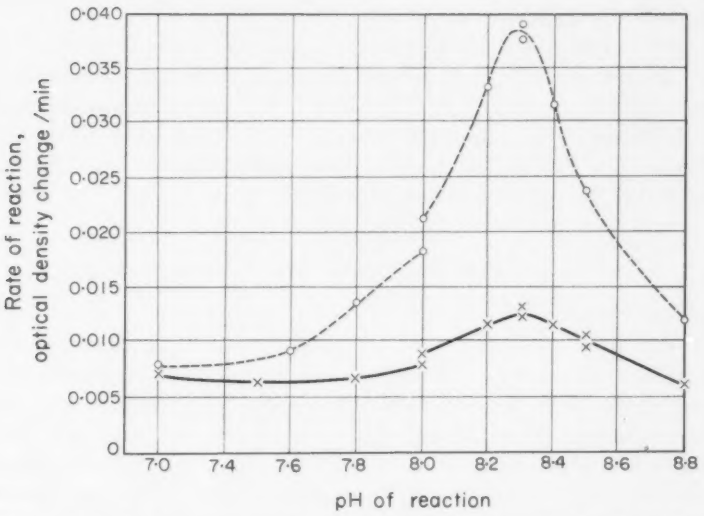

FIG. 3. The activity of glutamate–aspartate transaminase at various pH values in McIlvaine's (pH 7–8) and borate (pH 8–8·8) buffers. The reaction contained 0·5 ml of 0·1 M aspartate, 0·5 ml of 0·1 M α-ketoglutarate, 0·1 ml of enzyme solution and 3·9 ml of buffer. Pig-heart enzyme (X——X); roach enzyme (O- - - -O).

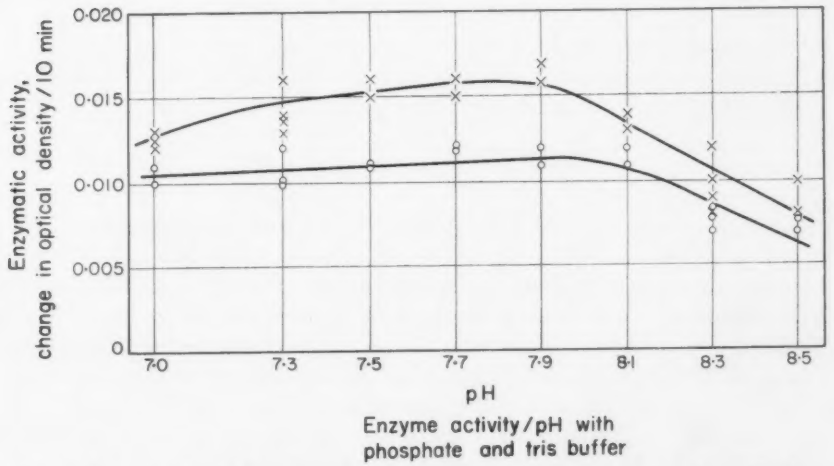

FIG. 4. The activity of glutamate–aspartate transaminase from roaches at various pH values in phosphate (X) and Tris (O) buffers. The reaction conditions were identical with those of Fig. 3.

borate buffer at more alkaline pH values. The results, given in Fig. 3, demonstrate a marked optimum at pH 8·3. In measuring the activity of roach enzyme with phosphate and Tris buffers no distinct optimum was observed (Fig. 4). The latter results are similar to those reported by Lichstein *et al.* (1945) using phosphate buffer in studying bacterial transaminase.

Cook (1956) lists numerous references to the effects of pH on the transaminase reactions; the results are often conflicting. Such a significant difference in activity in different buffers at the same pH suggested that the anions present affected the reaction. The following section presents evidence on the nature of this effect.

(e) *Equilibrium constant for the glutamate–aspartate reaction.* The equilibrium constant of the glutamate–aspartate reaction is given by:

$$K = \frac{(\text{oxaloacetate})^2}{[(\text{initial } \alpha\text{-ketoglutarate}) - (\text{oxaloacetate})]^2}$$

The standard reaction included 0·5 ml of 0·01 M aspartate and α-ketoglutarate in 3·7 ml of McIlvaine's or Clark and Lubs' buffer and 0·3 ml enzyme. This mixture reached an equilibrium at a measurable concentration of oxaloacetate. High enzyme concentrations were desirable in order to reach equilibrium rapidly and thus prevent undue decomposition of oxaloacetate (Green *et al.*, 1945).

The data in Table 2 indicate that at pH 8·3 the equilibrium constants for both the roach and pig heart enzyme were almost identical. Furthermore, these were quite different from the values determined at pH 7·3.

Similar results were obtained when the reaction was carried to equilibrium in the reverse direction, i.e. disappearance of oxaloacetate. In this case the equilibrium constant at pH 7·3 was 0·40 and at pH 8·3 it was 3·8, suggesting again that equilibrium favored the production of oxaloacetate at pH 8·3, but quite the reverse at pH 7·3.

This phenomenon was confirmed when the initial rates of transamination in both directions were measured. The equilibrium constant is also an expression of the ratio of the rates of a reversible reaction in both directions, i.e.

$$EK = \frac{\text{rate A}}{\text{rate B}} \quad \text{(see reaction (i))}$$

The equilibrium constant calculated from the initial rate measurements at pH 7·3 in McIlvaine's buffer was 0·38, and at pH 8·3 in borate buffer it was 1·41.

In order to test whether this difference in equilibrium was a function of the chemical nature of the buffer, the equilibrium of the reaction was also measured in phosphate buffer at pH 7·3 and 8·3. The values were 0·45 and 0·37 respectively. In Tris buffer the equilibrium constant at pH 7·3 was 0·47 and at pH 8·3 it was 0·40. None of the constants are appreciably different from one another or from that in McIlvaine's buffer. Furthermore, these are of the same order as values reported by Green *et al.* (1945), Krebs (1953) and Lichstein *et al.* (1945).

Since the equilibrium constant for a particular reaction is a fixed value regardless of enzyme concentration, substrate concentration or initial rate or reaction, it

was necessary to account for these large observed shifts. Several possibilities suggest themselves. For example, the difference may be due to a change in the *type* of reaction as a result of different buffers. The equilibrium may change also if one or more of the reactants and products are present in different ionic forms at

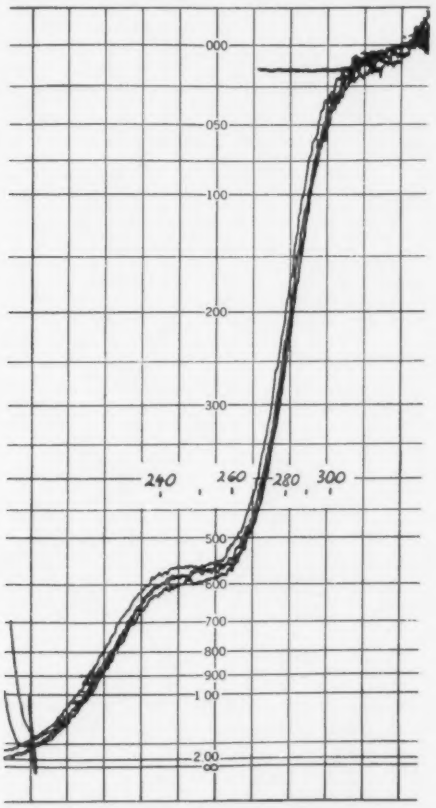

FIG. 5. Ultraviolet absorption spectra of oxaloacetate (0·5 mM) in Tris buffer, pH 6·0, 7·0, 8·0 and 9·0. The abscissa, wavelength in mμ; the ordinate, optical density.

different pH values. Or again, there may be a change in the equilibrium of two ionic forms of at least one of the components, i.e. oxaloacetate may exist in both the enol and keto forms.

In order to sort out some of these variables, the ultraviolet absorption spectra of the four components were determined at pH 7·3 and 8·3 in both McIlvaine's and borate buffers. The recording spectrophotometer Beckman model DK2 was used. The results demonstrated that the absorption characteristics of aspartate, glutamate and α-ketoglutarate were unchanged by the two buffers. Furthermore, the absorption spectrum of oxaloacetate in Tris buffer was the same at pH 6·0, 7·0, 8·0 and 9·0, as seen in Fig. 5. However, there was a marked difference

between the absorption spectrum of oxaloacetate at pH 7·3 in McIlvaine's buffer and that at pH 8·3 in borate buffer, suggesting that oxaloacetate in borate buffer had undergone some change (Fig. 6). It is well known that borate forms complexes with *cis*-diol structures (Zittle, 1951). Since the enol form of oxaloacetate would provide such a *cis*-diol structure at more alkaline pH values (Nossal, 1949), it is

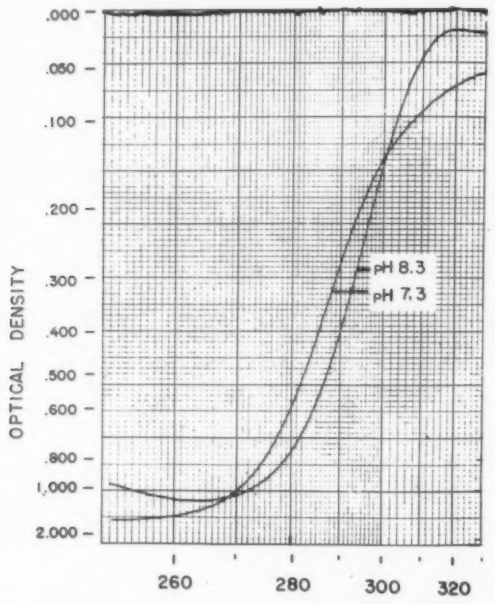

Fig. 6. Ultraviolet absorption spectra of oxaloacetate (2 mM) in McIlvaine's (pH 7·3) and borate (pH 8·3) buffers. Controls, seen at zero optical density, contained buffer only.

probable that borate complexes with oxaloacetate at pH 8·3, thus effectively removing it from the reaction mixture and hence shifting the equilibrium of the reaction in the observed direction. This phenomenon is most useful in the determination of the initial rates of reactions in which oxaloacetate is a product, therefore assays of glutamate–aspartate enzyme were carried out in borate buffer at pH 8·3.

(f) *Effect of temperature on glutamate–aspartate transamination.* The effect of temperature on the initial rate of glutamate–aspartate transamination catalysed by pig heart has been observed by Nisonoff & Barnes (1952) to be linear up to 40°C. They did not determine an "optimum" temperature, but their data suggest that it is above 57°C. Similar tests were made to determine the effect of temperature on roach enzyme. The reactions were carried out in spectrophotometric cells in a water bath and optical densities measured initially at 1 min and finally at 21 min.

The results of this experiment are presented in Fig. 7. It is apparent that the "optimum" temperature for roach enzyme does not exceed 48°C. An Arrhenius

TABLE 2—THE EQUILIBRIUM CONSTANT OF TRANSAMINATION IN SEVERAL BUFFERS

Buffer	pH	Equilibrium constant
Reaction (i, A)		
(a) Proceeding to equilibrium		
McIlvaine's	7·3	0·37 (with roach enzyme)
McIlvaine's	7·3	0·39 (with pig heart enzyme)
Borate	8·3	3·0 (with roach enzyme)
Borate	8·3	3·5 (with pig heart enzyme)
Reaction (i, B)		
(a) Proceeding to equilibrium		
McIlvaine's	7·3	0·40 (with roach enzyme)
Borate	8·3	3·80 (with roach enzyme)
(b) Initial rates method		
McIlvaine's	7·3	0·38 (with roach enzyme)
Borate	8·3	1·41 (with roach enzyme)

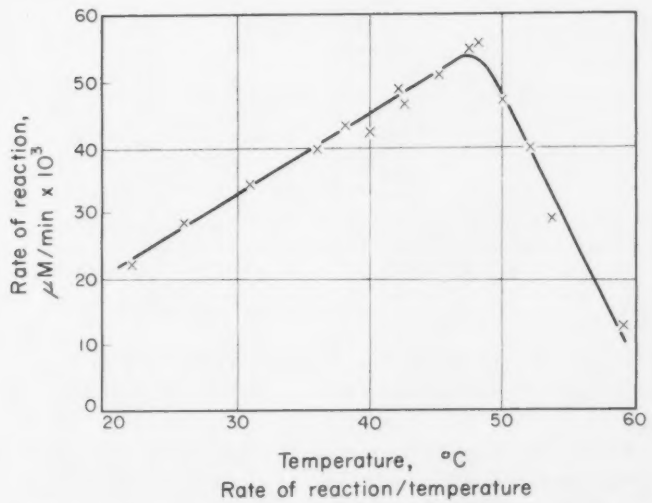

FIG. 7. The effect of temperature on purified glutamate–aspartate transminase from roach. The reaction consisted of 0·5 ml of 0·1 M α-ketoglutarate, 0·5 ml of 0·1 M aspartate, 3·9 ml of borate buffer, pH 8·3, and 0·1 ml of enzyme.

plot of these data indicates an activation energy of 6885 cal for this reaction in the temperature interval of 26–36°C, while Nisonoff & Barnes report a value of 12,500 cal for the reaction catalysed by pig heart enzyme.

2. Glutamate–alanine transamination

Two transamination reactions have been most extensively studied and are most active in organisms (Meister, 1955). One reaction has been considered; the other is the glutamate–alanine reaction. The method used to study the enzyme catalysing this reaction has been described above. Tests with roach homogenate yielded activities similar to those reported by Green *et al.* (1945) for pig heart enzyme, on the basis of weight of tissue.

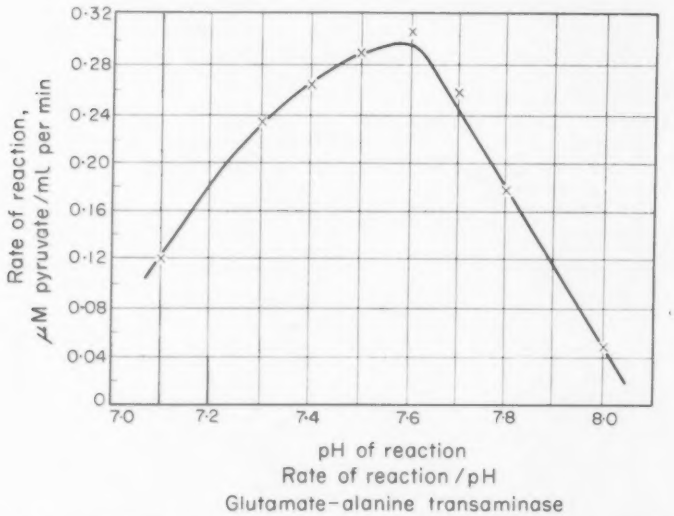

FIG. 8. The effect of pH on glutamate–alanine transaminase from roaches, using McIlvaine's buffer. The reaction consisted of 0·5 ml of 0·1 M α-ketoglutarate, 0·5 ml of 0·2 M D, L, α-alanine, 3·9 ml of McIlvaine's buffer, and 0·1 ml of homogenate.

The pH optimum of glutamate–alanine transaminase was measured in McIlvaine's buffer. The data presented in Fig. 8 show that reaction rate was greatest at pH 7·6.

The equilibrium constant of the glutamate–alanine reaction catalysed by roach homogenate was determined at pH 7·6. Reactions reached equilibrium after approximately 55 min. The concentration of pyruvate at equilibrium was 6·3 μM/ml, and the initial concentration of L-alanine was 10 μM/ml.

$$EK = \frac{(\text{pyruvate})^2}{(\text{alanine})^2} = 2{\cdot}9.$$

Cohen (1955) states that the values reported by others vary from 1 to 1·5. The racemization of D-alanine in the mixture may have affected the results.

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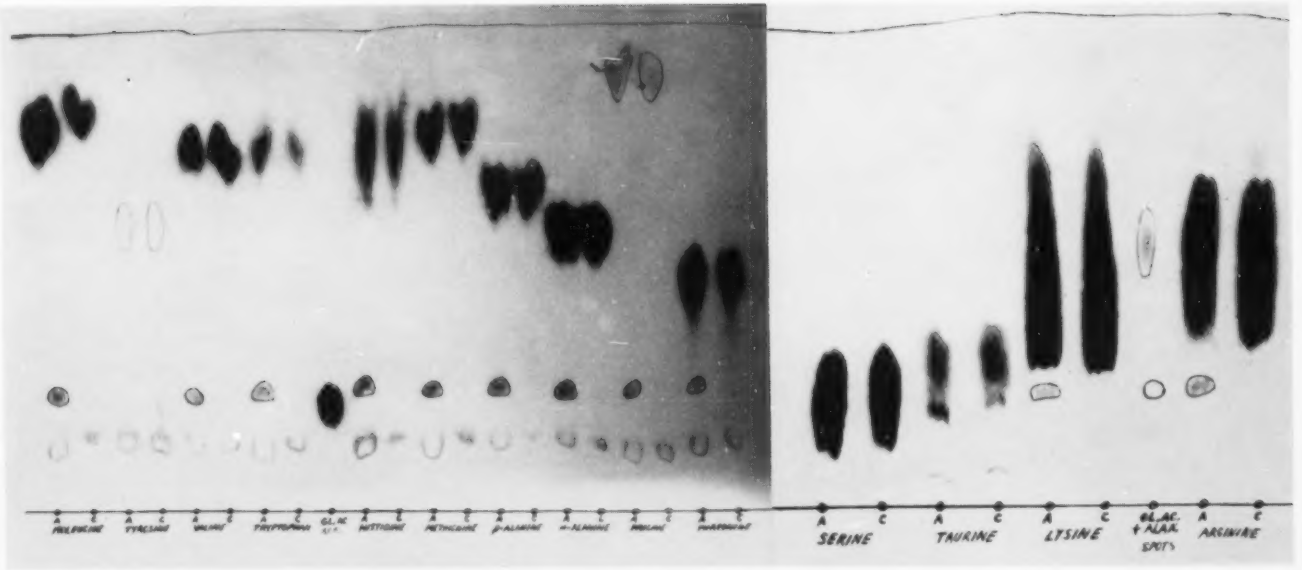

FIG. 9. Chromatograms showing the production of glutamic acid by most of the amino acids incubated with roach extract and α-ketoglutarate. The chromatograms were developed in 80 per cent phenol and dipped in 0·25 per cent ninhydrin in acetone. Active test – A, control, containing boiled homogenate, – C.

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3. *The extent of transamination in the roach*

Since it is known that most amino acids enter into the transaminase reaction generally reacting with α-ketoglutarate, some of the common amino acids were prepared as substrates for reaction with α-ketoglutarate catalysed by roach homogenate at pH 7·8.

Chromatography of the reaction mixtures (Fig. 9) showed the production of glutamate in reactions containing α-ketoglutarate and isoleucine, valine, tryptophan, histidine, methionine, β-alanine, α-alanine, proline, threonine, lysine, arginine and possibly taurine. Serine and taurine reactions were repeated using a butanol–acetic acid–water solvent, but no production of glutamate was observed. Kilby & Neville (1957) found that glycine, α-alanine, aspartate, leucine, valine, serine, threonine, tyrosine, phenylalanine, tryptophan, histidine, lysine, ornithine, arginine, cysteine, cystine and methionine transaminated with α-ketoglutarate, but that proline and hydroxyproline did not. Stetten & Schoenheimer (1944) demonstrated that labelled proline, fed to rats, was converted to glutamic acid and ornithine, and that the carbon chain as well as the nitrogen of proline was involved in the conversion. They concluded that this conversion was brought about by an oxidative opening of the pyrrolidine ring of proline. It is considered that the production of glutamate from proline in the presence of roach homogenate may have followed the same pathway.

4. *Glutamic dehydrogenase activity in the roach*

Amino acid and carbohydrate metabolism are related by the amination of α-ketoglutarate to produce glutamate. This pathway is catalysed by the enzyme glutamic dehydrogenase, which regulates the reaction:

$$\text{glutamate} + H_2O \underset{B}{\overset{\text{DPN} \quad \text{DPNH}}{\nwarrow A \nearrow}} \alpha\text{-ketoglutarate} + NH_4^+ + H^+. \qquad \text{(iii)}$$

Meister (1955) has considered the relationship and significance of transamination and the glutamic dehydrogenase reaction. He states that these coupled systems provide a mechanism for the oxidative deamination of L-amino acids, and that L-amino acid oxidases are of limited significance in most organisms.

Previously, Agrell (1949) demonstrated the oxidation of glutamate, postulating the presence of glutamic dehydrogenase in *Calliphora erythrocephala*, and Kilby & Neville (1957) have reported a glutamate–cytochrome *c* reductase system in *Schistocerca gregaria*. Since both transaminases and glutamic dehydrogenase compete for glutamate or α-ketoglutarate, and since the characteristics of the dehydrogenase reaction have a direct effect on the concentrations of amino acids, some of the properties of glutamic dehydrogenase in roaches were investigated.

Enzymic activity was measured after the method of Strecker (1955), as described above, by following the change in optical density at 340 mμ caused by a change in concentration of DPNH. The forward reaction mixture consisted of glutamate, DPN, homogenate and buffer, and the formation of DPNH was

measured. The reverse reaction mixture consisted of α-ketoglutarate, DPNH, ammonium chloride, amino acid-free homogenate and buffer, and the disappearance of DPNH was followed. It was necessary to remove amino acids from homogenate

Table 3—Glutamic dehydrogenase activity

Roach		Beef liver	
Initial rate	Equilibrium	Initial rate	Equilibrium
Change in optical density per 10 min	Total change in optical density	Change in optical density per 10 min	Total change in optical density
At pH 7·3 in McIlvaine's buffer			
0·003	0·005	0·013	0·023
0·003	0·007	0·012	0·022
At pH 7·3 in phosphate buffer			
0·003	0·006	0·017	0·023
0·003	0·007	0·019	0·020
0·002	—	0·018	—
At pH 8·3 in Clark and Lubs' buffer			
0·008	0·010	0·025	0·038
0·007	0·011	0·023	0·040
0·009	—	0·028	—
At pH 8·3 in phosphate buffer			
0·003	0·006	0·014	0·021
0·003	0·005	0·016	0·020
0·003	—	0·013	—

The above data refer to the deamination reaction (iii, A)*, the following refer to the amination reaction (iii, B)†, with roach enzyme.

0·032 at pH 7·3 in phosphate buffer
0·017 at pH 7·3 in phosphate buffer, half enzyme concentration
0·030 at pH 8·3 in phosphate buffer
0·030 at pH 8·3 in borate buffer

* Reaction A consisted of 0·3 ml of 0·1 M glutamate, 0·2 ml of 3 mM DPN, 2·4 ml of buffer and 0·1 ml of homogenate. Similar values were obtained in the presence of $1{\cdot}3 \times 10^{-3}$ M NaCN suggesting that the low glutamic dehydrogenase activities were not due to interference by DPNH oxidase activity.

† Reaction B consisted of 0·3 ml of 0·1 M α-ketoglutarate, 0·3 ml of 0·1 M NH_4C, 0·3 ml of 0·45 mM DPNH, 0·3 ml of homogenate and 1·8 ml of buffer. Temperature, 22°C. All values were corrected for DPNH oxidase activity as measured in the controls. Under these conditions the DPNH oxidase activity was very low, producing an average change in optical density of about 0·003 in 10 min.

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of roach fat body to obtain any appreciable dehydrogenase activity in the reverse reaction. It was considered that these amino acids transaminated with the substrate, α-ketoglutarate, thus decreasing the rate of the dehydrogenase reaction.

The extent of glutamic dehydrogenase activity in borate and McIlvaine's buffers was compared with the activity in phosphate buffer at pH 7·3 and 8·3. Beef liver (250 mg/ml) and cockroach fat body (100 mg/ml) were used as sources of enzyme. From the results summarized in Table 3, it is apparent that enzyme from both organisms is as active in McIlvaine's as in phosphate buffer at pH 7·3, but appears much more active in Clark and Lubs' than in phosphate buffer at

TABLE 4—EQUILIBRIUM CONSTANTS* OF THE GLUTAMIC DEHYDROGENASE REACTION

pH	Buffer	Equilibrium constant
Roach enzyme		
7·3	McIlvaine's	$0{\cdot}23 \times 10^{-19}$
7·3	Phosphate	$0{\cdot}25 \times 10^{-19}$
8·3	Clark and Lubs' (borate)	$0{\cdot}11 \times 10^{-19}$
8·3	Phosphate	$0{\cdot}25 \times 10^{-20}$
Beef liver enzyme		
7·3	McIlvaine's	$9{\cdot}9 \times 10^{-19}$
7·3	Phosphate	$9{\cdot}8 \times 10^{-19}$
8·3	Clark and Lubs'	$6{\cdot}3 \times 10^{-19}$
8·3	Phosphate	$9{\cdot}6 \times 10^{-20}$

* Constants are calculated from the data presented in Table 3.

pH 8·3. This situation is analogous to that for transaminase activity. However, the reverse reaction is not appreciably affected by borate buffer. As already indicated, the low activity of glutamic dehydrogenase in the fat body of cockroaches is not due to interference by DPNH oxidase. The activity of the DPNH oxidase system in our insect preparations was very low and in many preparations could not be detected. Values for the equilibrium constant of the glutamic dehydrogenase reaction are given in Table 4, as calculated for the forward reaction (iii). The equilibrium changes with pH as expected (Strecker, 1955), and with borate buffer, possibly in a manner similar to that observed for transamination. Three points should be noted especially: the activity of roach fat body homogenates was less than that of beef liver on the basis of tissue weight; the glutamic dehydrogenase reaction is an amination reaction, the concentration of glutamate being much greater at equilibrium than the concentration of α-ketoglutarate; the equilibrium of the reaction using roach fat body favors amination even more markedly than the equilibrium of the reaction with beef liver. This difference in the equilibria may be the result of other reactions affecting net equilibrium, but in any case the results are significantly different.

The effect of glutamate concentration on glutamic dehydrogenase. The effect of varying glutamate concentration on the activity of glutamic dehydrogenase in homogenates of roach fat body and beef liver was determined by measuring the rates of reactions containing various concentrations of glutamate and catalysed by equal amounts of enzymic activity. The results are given in Fig. 10, in which rates of reactions are plotted against concentrations. It is apparent that roach enzyme

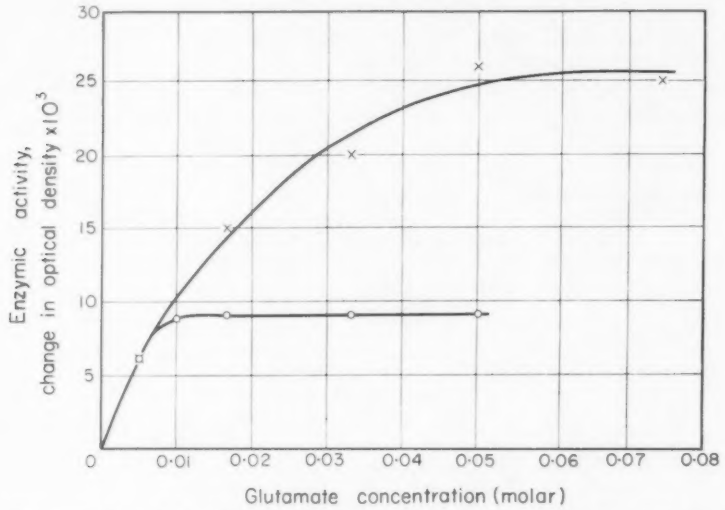

FIG. 10. Comparison of the affinity for glutamate of glutamic dehydrogenase from roach fat body (O) and beef liver (X). The reaction contained 0·3 ml of glutamate at ten times the concentrations expressed on the abscissa, 0·1 ml of homogenate, 0·2 ml of 3 mM DPN and 2·4 ml of borate buffer, pH 8·3.

has a smaller Michaelis' constant, 0·004 M/l, than beef liver, 0·013 M/l. From such evidence, it was concluded that glutamic dehydrogenase from roach fat body differs from this enzyme in beef liver. Because of its higher affinity for glutamate, the activity of glutamic dehydrogenase in roach fat body may affect the rate of deamination at a much lower substrate concentration than does the enzyme in beef liver.

Transamination versus amination. The previous experiments raised the question of the degree of interaction and competition for substrates between transaminase and glutamic dehydrogenase in the roach. An experiment devised in an attempt to determine the relationship of these two pathways in the roach consisted of determining the rate of transamination catalysed by fat body homogenate in reaction (i) B, (glutamate + oxaloacetate $\rightleftharpoons$ α-ketoglutarate + aspartate) with and without DPN being present. At pH 7·3 there was a marked (72 per cent) decrease in transamination in the presence of DPN. It was assumed that the presence of DPN stimulated the dehydrogenase reaction, thus removing glutamate from the reaction

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and producing α-ketoglutarate, which would cause the transamination reaction to reach equilibrium more quickly than otherwise. Previous evidence has shown the effects of amino acids on the rate of amination of α-ketoglutarate; the reaction simply did not proceed. From these results it was concluded that glutamate–aspartate transaminase and glutamic dehydrogenase compete very actively for substrates, and possibly that the equilibrium of each is affected by the other system *in vivo* in the roach.

DISCUSSION

The results of many studies have indicated that transaminases from bacteria, plants and vertebrates have marked similarities. The present study attempts to relate the properties of transaminases found in roaches to those of transaminases of mammalian tissue.

The comparison of roach transaminase with pig heart enzyme and with transaminases reported by other workers suggests that these enzymes are similar in chemical properties and action. Quantitative but not qualitative differences were noticed in the purification of glutamate–aspartate enzyme from roaches and pig heart by the same procedure. Moreover, the activities of whole homogenates of these two materials are of the same order of the basis of weight of tissue, and the behaviour of pig heart and beef liver enzymes parallels that of roach enzyme with regard to optimum pH, buffer effects and substrate affinity.

The resolution of apoenzyme and coenzyme, and the reactivation of the protein fraction of roach transaminase by incubation with pyridoxal-5-phosphate is clear evidence of the role of vitamin B_6 as coenzyme in transamination in the roach. Kilby & Neville (1957) also demonstrated that pyridoxal-5-phosphate reactivated resolved enzyme preparations of the locust fat body, and concluded that vitamin B_6 acted as coenzyme in insect transaminases. They report that the amounts of glutamate–aspartate and glutamate–alanine enzymes in locust fat body are of the order of those found for rat liver slices. Previously, Barron & Tahmisian (1948), in studying the metabolism of cockroach muscle using manometric techniques, had observed that the level of glutamate–alanine enzyme in this tissue was similar to values reported for mammalian tissues. Further, the number of amino acids involved in transamination in insects, demonstrated chromatographically in both locust and roach tissues, suggests that most amino acids in insects transaminate. Therefore, it seems reasonable to conclude that transamination, which is similar in bacteria, plants and vertebrates with respect to extent, distribution, coenzyme and kinetic characteristics is of the same significance in insects.

Wigglesworth (1950) emphasizes that the concentration of amino acids in insect hemolymph is 50–100 times that of mammalian blood; possibly increased amination of carbohydrates (keto acids) and their subsequent transamination account for these high concentrations. It must be emphasized that glutamic dehydrogenase is not primarily a "deaminating" enzyme, but that equilibrium favors the amination of α-ketoglutarate, and in turn the transamination equilibrium for the glutamate–aspartate reaction favors the disappearance of glutamate. Thus the equilibria of at

least these two important reactions tend to maintain high levels of amino acids. Certainly the relative amounts and kind of amino acids in the roach may to some degree be regulated by the transaminase reactions, since only this conclusion can explain the increase in glutamine and glutamic acid in roach blood observed on feeding *Periplaneta americana* large amounts of alanine and α-ketoglutarate (Patton, 1953).

Since the transaminase–glutamic dehydrogenase system is considered to be the main pathway for the deamination of amino acids (Meister, 1955), the activity of glutamic dehydrogenase may be the rate-limiting step which regulates the level of the amino acid pool. The evidence that roach glutamic dehydrogenase has a smaller Michaelis' constant than beef liver enzyme, that the activity of this enzyme is present in the fat body (a most predominating tissue in insects), and that the equilibrium of the reaction catalysed by roach enzyme favors amination of α-ketoglutarate explains, at least in part, the high amino acid content of roach hemolymph.

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THE POSSIBLE ROLE OF 5-HYDROXYTRYPTAMINE IN THE MOTOR ACTIVITY OF EMBRYOS OF SOME MARINE GASTROPODS

Kh. S. KOSHTOYANTS, G. A. BUZNIKOV and B. N. MANUKHIN

Institute of Animal Morphology, Academy of Sciences of the USSR, Moscow

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Abstract—1. 5-hydroxytryptamine (5-HT) stimulates the beats of prototrochal and velar cilia of nudibranch embryos. The ability for reacting to 5-HT appears in the embryos at the trochophore stage and is retained up to metamorphosis.

2. Most sensitive to 5-HT are veligers with a well-developed velum but with no shell (threshold concentration 10^{-16} g/ml). At later stages the sensitivity to 5-HT decreases presumably due to the onset of inhibitory influences of nervous centres.

3. Isolation of velar cells restores their high sensitivity to 5-HT.

4. 5-HT is assumed to be an endogenous factor inducing ciliar beats of the prototroch and velum in Nudibranchia.

5. A new biological method of quantitative assay of 5-HT is proposed (sensitivity 10^{-16} g/ml and $5 \cdot 10^{-12}$ μg) with intact embryos and isolated velar cells of Nudibranchia as test-objects.

INTRODUCTION

THE occurrence, metabolism and physiological significance of 5-hydroxytryptamine (serotonin, 5-HT) have been most extensively studied in higher vertebrates. The comparative-physiological aspect of 5-HT has been studied in less detail although the available evidence suggests its important role in many invertebrates. 5-HT has been discovered in coelenterates, molluscs, crustaceans, insects and ascidians (Erspamer, 1954, 1957; Kerkut & Laverack, 1958; Page, 1958). 5-HT presumably acts as a mediator of extracardiac nerves in molluscs (Erspamer & Ghiretti, 1951; Florey & Florey, 1953, 1954; Welsh, 1954, 1956, 1957; Fänge, 1955; Koshtoyants, 1957a; Meng, 1958). In decapods, of similar significance is a related compound, viz. 5,6-dihydroxytryptamine (Carlisle, 1956). 5-HT which is apparently released in the nerve endings causes relaxation of smooth muscles in molluscs (Twarog, 1954, 1960; Fänge & Mattisson, 1958). The role of 5-HT present in the ganglia of molluscs and crustaceans is obscure as yet (Welsh, 1957; Florey & Florey, 1954). In cephalopods it is synthesized in the posterior salivary glands whence it passes to the hemolymph or is released with the venom of these glands. The toxic secretion of the hypobranchial body of Muricidae likewise contains 5-HT (Erspamer & Ghiretti, 1951; Kahr, 1958). It will be noted in this connexion that 5-HT is an important component of the venom of cnidoblasts in coelenterates and of skin glands of amphibians, of the scorpion and wasp venom as well as of stinging hairs of nettle (Jacques & Schachter, 1954; Chesher & Collier, 1955; Erspamer, 1957; Adam & Weiss, 1959).

The possible role of 5-HT in the physiological processes in the vertebrate and invertebrate embryos has been practically neglected, although it is interesting not only *per se*, but may also contribute to the elucidation of the development of regulatory mechanism during ontogenesis.

MATERIALS AND METHOD

To elucidate the physiological role of 5-HT in embryos, experiments were carried out on eggs of Nudibranchia. Since preliminary analyses showed a high content of 5-HT in the tissues of adult Nudibranchia it seemed probable that this substance may be available in embryos as well. Nudibranch ova are available throughout the summer season. A great number of synchronously developing embryos can readily be obtained in aquaria.

The study was carried out at the Marine Biological Station of the Moscow University (White Sea). Seven nudibranch species were examined, viz. *Dendronotus frondosus*, *Coryphella rufibranchialis*, *Acanthodoris pilosa*, *Aeolidia papillosa*, *Onchidoris muricata*, *Cadlina laevis* and *Cuthona* sp. (*concinna* ?).

Individual experiments were also carried out on the prosobranch *Lacuna divaricata*. The development of all the above species, except *Cadlina laevis*, involves the formation of a motile larva of the veliger type. The *C. laevis* embryos do not pass the veliger stage and locomotion is effected by a foot invested with ciliated epithelium, and not by velum. The experimental material was incubated at a temperature up to 10–15°C. The experimental procedure was a very simple one. Intact embryos or isolated velar cells were placed in sea water solutions of 5-HT, LSD-25, acetylcholine, adrenaline or noradrenaline. The velar cells were isolated by a 30–60 sec homogenization of the embryos at a low temperature. The effect of the above substances was estimated visually. Observations were made at a low amplification of the microscope in diffuse light. Photomicrography was used for morphological control.

EXPERIMENTAL RESULTS

1. *Effect of 5-HT upon the motor activity of embryos*

5-HT greatly accelerates and enhances the beats of the prototrochal and velar cilia of all nudibranch species endowed with these embryonic locomotor organs. The sensitivity of the embryos to 5-HT changes in the course of development (Fig. 1). The embryos begin to react to 5-HT at the trochophore stage. The motor activity of these embryos consists of weak pushes caused by the activity of the prototrochal cilia. 5-HT (10^{-6}–10^{-12} g/ml) induces rotary movements of the embryos, and in species with relatively greater perivitelline spaces (*A. papillosa*) swimming inside the egg capsules.

Further development of the embryos up to the early veliger stage (Fig. 2a) is accompanied by an increase in sensitivity to 5-HT which attains its maximum in veligers still devoid of shells but with well-developed velar lobes (Figs. 1, 2b). At this stage, the embryos are slowly rotating owing to the continuous activity of

velar cilia. 5-HT (10^{-10}–10^{-16} g/ml) greatly increases the rate of rotation. At higher concentrations (10^{-5}–10^{-9} g/ml) this reaction is supplemented by a very characteristic tremor of the rotating embryos.

Subsequently, the sensitivity to 5-HT begins to decrease (Fig. 1), concurrently with substantial changes in the motor function of the embryos and in their morphological status. The relative size of velar lobes diminishes, and there appears a

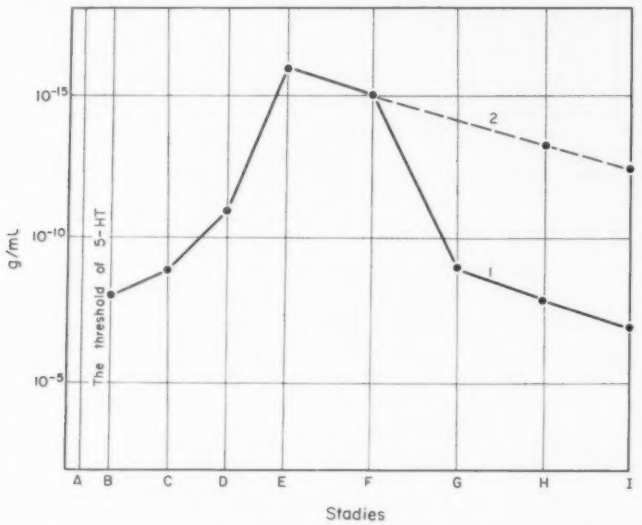

FIG. 1. Age changes in sensitivity to 5-HT in embryos (1) and in isolated velar cells (2) of *Dendronotus frondosus*. A—trochophore, B—trochophore, C—beginning of formation of velum, D—early veliger, E—veliger without shell, F—veliger with a small shell, G—veliger with a well-developed shell, H—veliger prior to hatching, I—veliger after hatching.

shell which gradually increases (Fig. 2c). Ciliar beats are alternated with more or less long pauses during which the velar lobes shrink owing to the contraction of the velar retractor. According to Carter (1926), the appearance of pauses in the ciliar activity is linked with the growth of nerve fibres towards velar cells. Soon after hatching the nudibranch embryos leave the shell. 5-HT continues to affect the motor activity of such embryos probably until the loss of the velum.

The effect of 5-HT upon ciliar beats is very steady. It lasts not less than 2–3 days upon long exposure of the embryos to 5-HT solutions. The sensitivity of various species of nudibranch embryos to 5-HT is different. Among the species studied, the embryos of *C. rufibranchialis* proved to be most sensitive.

2. *The effect of* 5-*HT upon the motor activity of isolated velar cells*

5-HT stimulates the motor function not only of intact embryos but of isolated velar cells as well. Ciliary beats of these cells in sea water last as long as 1–3 hr

after isolation. 5-HT increases the background activity of velar cells and restores and enhances the beats of the arrested cilia. The sensitivity of isolated velar cells to 5-HT is approximately similar to that of intact embryos at early stages of development. At later stages it decreases in intact embryos while remaining high in isolated velar cells (Fig. 1). Thus, the threshold concentration of 5-HT in intact late veligers of *Cuthona* sp. equals 5×10^{-8} g/ml, and in isolated velar cells 1×10^{-12} g/ml.

It has been shown on isolated foot fragments of a *Cuthona* sp. veliger that the cilia of the ciliated epithelium do not react to 5-HT. In this connexion it seems worth while noting that in *C. laevis* embryos which lack any velum, 5-HT stimulates the activity of the ciliated epithelium of the foot.

3. *Specific features of the reaction of embryos to 5-HT*

The specificity of the reaction to 5-HT was checked in two ways: (i) by testing the effect of other physiologically active compounds upon the motor function of the embryos, and (ii) by means of the antiserotonin preparation—LSD. It was found that acetylcholine, adrenaline and noradrenaline with a concentration of

TABLE 1—EFFECT OF 5-HT UPON MOTOR INHIBITION CAUSED BY LSD ($2{\cdot}5 \times 10^{-5}$ g/ml)

	Intact embryos		Isolated velar cells	
	Prior to LSD	After LSD	Prior to LSD	After LSD
Sea water	+ + +	0	+ + +	+
5-HT (g/ml) 10^{-10}	+ + +	+	+ + + +	+ + +
10^{-9}	+ + +	+ +	+ + + + +	+ + + +
10^{-8}	+ + + +	+ + +	+ + + + + +	+ + + + + +
10^{-7}	+ + + + + +	+ + +	+ + + + + +	+ + + + + +

10^{-4} g/ml or less do not affect the motor function of intact embryos. The results obtained with LSD on late veligers of *Cuthona* sp. are summarized in Table 1. LSD inhibits spontaneous movements of intact embryos up to complete paralysis of the cilia without affecting the periodicity of contractions of the velar retractor. 5-HT restores the background activity of the veliger inhibited by LSD, and when applied beforehand it prevents the effect of LSD. This protective action is apparent even at such concentrations of 5-HT which by themselves do not affect the motor function. LSD also inhibits the spontaneous movements of isolated velar cells. However, in contrast to intact embryos, ciliar beats are not completely arrested. 5-HT disinhibits the inhibition caused by LSD in this case as well and likewise exhibits a protective effect. The effective concentrations of 5-HT proved much inferior to those found in intact embryos.

At earlier developmental stages of *Cuthona* sp. the effect of LSD exhibits two phases, viz. inhibition of ciliar beats rapidly followed by a serotonin-like effect.

In early and middle veligers of *D. frondosus* and *A. pilosa* LSD (10^{-5}–10^{-7} g/ml) exerts a serotonin-like stimulating effect without any preliminary inhibitory phase. Thus, species and age specificity of the reactions of nudibranch embryos to LSD become apparent. It will be noted, in conclusion, that the above concentrations of LSD do not affect ciliary activity of the ciliated epithelium of the veliger foot in *Cuthona* sp. and activates ciliary beats of the foot of *C. laevis* embryos.

4. *Nudibranch embryos as test-objects for assaying* 5-*HT*

In view of the exceptionally high sensitivity of nudibranch embryos to 5-HT they can be used for determination of this substance. The following variants of the method are suggested in accordance with the age peculiarities of the reactions of embryos to 5-HT (Fig. 1).

Variant 1. *Early trochophores or early veligers* (*Fig.* 2a). 5-HT causes rotation of the embryos. The strength of the reaction to 5-HT is determined from the average number of rotations per minute 5–10 min after the addition of a drop of the solution to be tested. This value depends on the concentration of 5-HT which makes possible plotting of a calibration curve. This variant is convenient for a quantitative assay of 5-HT within the range of 10^{-6}–10^{-12} g/ml.

Variant 2. *Veliger stage with no shell, velar lobes well developed* (*Fig.* 2b). 5-HT greatly accelerates rotation of the embryos. A calibration curve can likewise be plotted. Owing to the high background activity of the embryos this variant is less convenient for quantitative purposes. Its advantage is very high sensitivity (threshold concentration of 5-HT below 10^{-16} g/ml and the minimal quantity of 5-HT to be determined—5×10^{-12} μg).

Variant 3. *Veliger stage with a most developed shell* (*Fig.* 2c). Isolated velar cells are used as test-objects which, in contradistinction to intact embryos, retain high sensitivity to 5-HT (Fig. 1). The variant is convenient for checking the specificity of the serotonin-like effect of the solutions to be tested, since use can be made of the antiserotonin preparation LSD.

A more detailed description of this method for 5-HT assay is presented elsewhere (Manukhin & Buznikov, 1960).

DISCUSSION

In their morphology and ciliar activity the motor cells of the velum are very similar to those of the ciliated epithelium (Carter, 1926, 1928). However, the chemical basis of motor activity of velar cells is of a peculiar character. Spontaneous rhythmicity of the ciliary apparatus in all cases so far studied is linked with the system "choline acetylase–acetylcholine = cholinesterase". Acetylcholine may be considered as a local hormone inducing movement in such diverse objects as cilia of the protists, ciliated apparatus of tentacles of sea anemones, ciliated epithelium of the gills in mussels, frog oesophagus and rabbit trachea, cilia of sea urchin embryos, etc. (Burn, 1956; Koshtoyants, 1957b). In the present case acetylcholine does not affect ciliary beats. In addition, lack of acetylcholine esterase has been demonstrated histochemically in velar cells of almost all the

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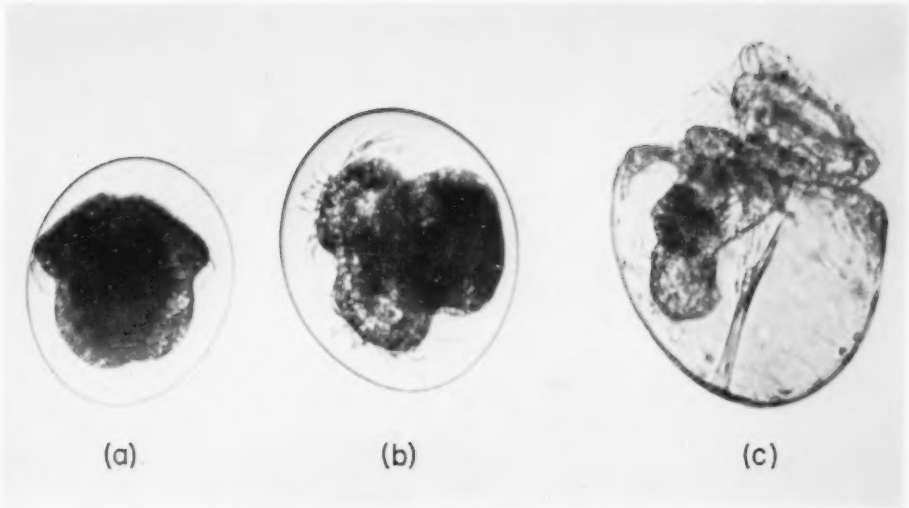

FIG. 2. Embryos of *D. frondosus* at various stages of development: (a)—early veliger, (b)—veliger without shell, (c)—veliger prior to hatching.

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nudibranchia studied (Buznikov, 1960). Whereas small concentrations of 5-HT enhance the spontaneous motor function of velar cells and restore the beats of the arrested cilia, the antiserotonin preparation LSD counteracts the effect of 5-HT and inhibits the spontaneous motor activity of intact embryos and of isolated velar cells. It may thus be suggested that 5-HT or some related compound functions as a local hormone which incites the movements of prototrochal and velar cilia of nudibranch embryos.

Ciliar activity of the prototroch or velum of veligers of other molluscs and of polychaete trochophores might likewise be linked with 5-HT. It has been shown, in particular, that 5-HT accelerates ciliar beats in the velum of the *Lacuna divaricata* embryos (Prosobranchia). In some Nudibranchia, e.g. *Cuthona* sp., along with the velum, locomotion in embryos is effected by the foot which is invested with ciliated epithelium. 5-HT and LSD do not influence the activity of the cilia of the foot. This is suggestive of a difference in the biochemical mechanism underlying the motor function of the ciliated epithelium and of velum in nudibranch embryos. However, in *C. laevis*, which does not develop any velum, the foot cilia distinctly react to 5-HT and LSD.

The reaction of the embryos to 5-HT commences at the trochophore stage. The subsequent increase in sensitivity to 5-HT can be accounted for by the continued formation of the respective receptors localized in the velar cells. The change in the receptive structures is also indicated by the fact that the antimetabolite of serotonin, LSD, calls forth ciliary beats at early stages of development but inhibits the motor activity in late veligers. The drop in sensitivity to 5-HT as noted during the second half of embryogenesis is not linked with any changes in the velar cells themselves. Isolated velar cells retain their capacity to react to low concentrations of 5-HT. This drop in sensitivity is presumably due to the establishment of central inhibitory influences upon ciliary activity. It is well known that innervation of velar cells appears precisely at the stages when, according to our data, the sensitivity of the embryos to 5-HT begins to decrease (Carter, 1926). The chemical factor mediating the central inhibitory influences does not seem to be identical to acetylcholine since the latter does not influence the motor function of the embryos.

The role of 5-HT as an agent inciting the motor activity disappears simultaneously with the loss of the velum. In adult Nudibranchia as in other gastropods 5-HT is presumably one of the mediators of the nervous system. Thus, during ontogenesis of Nudibranchia, the physiological role of 5-HT undergoes an appreciable change.

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FURTHER STUDIES ON THE EFFECT OF MAMMALIAN PARATHYROID EXTRACT ON THE SERUM CALCIUM LEVELS OF TWO CLOSELY RELATED TELEOSTS

W. R. FLEMING and A. H. MEIER

Zoology Department, University of Missouri, Columbia, Missouri

(*Received* 7 *November* 1960)

Abstract—1. The effect of mammalian PTH on the turnover of serum calcium, as judged by Ca^{45} assay, on *Fundulus catenatus* during the breeding season, and on *Fundulus kansae* during the post-breeding season has been studied.

2. *F. catenatus* and the male *F. kansae* did not respond to PTH; female *F. kansae* which had been exposed to estradiol benzoate showed decreased serum Ca following PTH injections.

3. It is concluded that *F. catenatus* does not respond to PTH at any time, and that the response found in the female *F. kansae* is probably dependent upon estrogen conditioning.

INTRODUCTION

We have recently reported that gravid *Fundulus kansae* showed an increased turnover of serum calcium following an injection of mammalian parathyroid extract (Fleming & Meier, 1961). We also reported that the male *F. kansae* gave only a slight response of doubtful significance, and that we had not been able to show a response in the closely related *F. catenatus*. We suggested that the positive response to PTH depended in part on the level of circulating estrogens, since the female *F. kansae* responded during the breeding season but did not do so later on in the year. At that time, we had not had the opportunity to study the effect of PTH on the serum calcium levels of *F. catenatus* during its rather short breeding season, nor had we examined the effect of prolonged estrogen treatment on *F. kansae* during the post-breeding period. This paper reports the results of such studies.

MATERIALS AND METHODS

Fundulus kansae, the plains killifish, were collected from a salt spring "Booneslick" in late October, approximately 6 weeks past the end of the breeding season. They were held in large concrete holding tanks for 1 week and then transferred to a constant temperature room held at $16 \pm 0{\cdot}5°C$. Twenty fish weighing between 1·1 and 1·3 g were placed in small aquaria containing 4 l. of tap-water (chlorine free). Following a 2 day equilibration period, each fish was injected intraperitoneally with a 0·17 M saline solution containing Ca^{45}. Each fish received 1 mc of the isotope in 0·05 ml of saline. At the same time, 1 mg of estradiol benzoate (Nutritional Biochemicals Corporation) was added to the water of alternate aquaria.

Following a 21 day equilibration period to the isotope and hormone, the water was changed in all aquaria and eight experimental battery jars containing 2 l. of water were set up. 0·5 mg of estradiol benzoate was added to the jars numbered 5, 6, 7 and 8. Eight fish were transferred to each jar and care was taken to see that only fish

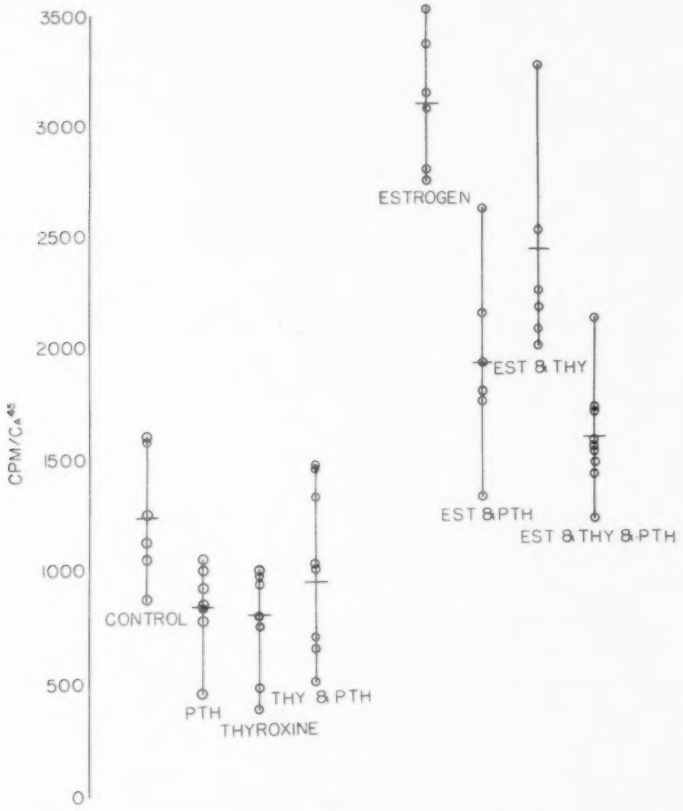

FIG. 1. The effect of mammalian PTH, thyroxine, estradiol benzoate, and combinations of these hormones on the serum Ca^{45} levels of female *F. kansae*. Each point represents a single animal and gives the radioactivity of a 10 μl sample of serum.

which had been exposed to the estrogen were placed in the jars to which estradiol benzoate had been added. The fish in the jars numbered 3 and 4, and 7 and 8 were injected with thyroxine (6 μg in 0·02 ml saline). The fish in the remaining jars were injected with saline alone. 1 week later all the fish except those in jars 1 and 5 were injected with 1 I.U. of mammalian PTH. 12 hr later the animals were sacrificed and the serum assayed for Ca^{45}. The experiment was carried out on the males and females separately.

Fundulus catenatus, the Ozark studfish, were collected from the Sinking River, south of Salem, Missouri, in late May and early June. Only males in full breeding color and females carrying eggs were used.

The details of the collection of serum and the radioisotope assay have been described previously (Fleming & Meier, 1961).

RESULTS

The results obtained support those reported previously. *Fundulus catenatus* failed to show any response to mammalian PTH, as judged by Ca^{45} turnover, and the same was true of the male *F. kansae*. Since no positive results were obtained, no data are presented here.

The female *F. kansae*, on the other hand, responded readily to the hormone providing that they had been previously exposed to estradiol benzoate. The results of these experiments are shown in Fig. 1.

DISCUSSION

From Fig. 1 it can be seen that in the absence of estradiol benzoate, neither PTH, thyroxine, or a combination of the two, have any effect on serum calcium levels, as judged by Ca^{45} turnover. In the presence of the estrogen the serum calcium levels are elevated, and PTH injection is followed by a drop in serum radioactivity.

We chose to test the effect of thyroxine since we have found that this hormone enhances the response to estrogens in the male *F. kansae* (unpublished data). The results presented here suggest that thyroxine may have an antagonistic effect, however, and require further investigation.

From the data presented here and from those reported in our first paper, we conclude that we have in these two closely related species both a sex and species difference in response to mammalian PTH. At the present time we are unable to explain why only the female *F. kansae* respond to PTH. Clearly, further studies on the calcium metabolism of these teleosts are indicated.

We do feel that the data presented here lend support to our suggestion that the response found in the female *F. kansae* may be conditioned, in part, by the level of circulating estrogens. We must point out, however, that we have not attempted to make any such measurements, nor do we at this time have any information on the biochemical fate of the estradiol benzoate added to the aquarium water.

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METABOLISM OF GLUCOSE IN THE CRABS *CANCER MAGISTER* AND *HEMIGRAPSUS NUDUS**

V. R. MEENAKSHI† and B. T. SCHEER

Department of Biology, University of Oregon

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Abstract—1. The major blood carbohydrates of crabs include glucose, maltose, maltotriose, maltotetrose and glucose-6-phosphate. No trehalose could be detected. There are considerable amounts of acid-soluble glycoproteins.

2. The maltose oligosaccharides show marked variation with diet and season; the glucose level is relatively stable.

3. The changes in blood composition following feeding or injection of glucose suggest that glucose-6-phosphate is an intermediate in the synthesis of maltose oligosaccharides and that the latter are intermediates in glycogen synthesis. The carbon of labeled glucose appears first in glucose-6-phosphate, then in the oligosaccharides and finally in glycogen.

4. The carbon of labeled maltose appears relatively early in glycogen but is not recovered from glucose within 48 hr. This appears to confirm the sequence postulated above.

5. Glucose carbon is incorporated into chitin during the early post-moult period, but not during intermoult.

6. Glucose carbon is incorporated into mucopolysaccharide in the hepatopancreas at all stages but appears in the mucopolysaccharide of the integument only during the postmoult stages, not during the intermoult or premoult stages.

7. Radioactive carbon from maltose appears in the respiratory CO_2 when only maltose is administered. If labeled maltose and unlabeled glucose are administered together, the fraction of maltose which appears as CO_2 decreases in proportion to the amount of glucose administered.

8. A general hypothetical scheme of carbohydrate metabolism in crustaceans is presented and it is suggested that glycogenesis occurs through the UDPG–Synthetase system while glycogenolysis involves phosphorylase and the two paths are essentially separate.

INTRODUCTION

STUDIES on carbohydrate metabolism in crustaceans began with the demonstration of glycogen in the hepatopancreas of a crustacean by Bernard (1879). Observations on the presence of reducing substances in the blood are numerous. Hemmingsen (1925) found that glucose disappears more rapidly than it can be oxidized, and considered this to indicate the existence of a regulatory mechanism. Verne (1924) showed that glycogen accumulates in the integumentary tissue prior to moulting

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† Present address: c/o V. R. Krishna Iyer Advocate, Hospital Road, Ernakulam, Kerala State, India.

but disappears as chitin is being formed and hence concluded that glycogen is a precursor of chitin. Renaud (1949) confirmed this observation, and followed the changes in concentration of the major carbohydrates through the intermoult cycle. Hu (1958) observed that in the crab *Hemigrapsus nudus* there is a polysaccharide other than glycogen which on hydrolysis yields glucose, galactose and fucose, and which has probably been included in glycogen estimations heretofore. Meenakshi & Scheer (1959) showed that this mucopolysaccharide is present in the hepatopancreas and integument of *Hemigrapsus nudus* and migrates as a single component in an electric field.

Though the studies on blood sugar are numerous, until recently there was no means of establishing that the values obtained as blood sugar represent glucose or any single substance. Studies using the fermentation method clearly indicate that a major fraction of the reducing substance is not glucose (Kleinholz & Little, 1949; Scheer & Scheer, 1951). McWhinnie & Scheer (1958) have shown that the actual glucose value is only about 20 per cent of the total blood sugar in the shore crab *Hemigrapsus nudus*. Riegel (1960) finds somewhat higher values in the crayfish *Pacifastacus leniusculus*. Hu (1958) showed the presence of free glucosyl oligosaccharides in *Hemigrapsus nudus*. McWhinnie & Saller (1960) have identified the presence of glucose, maltose, maltotriose, maltotetrose and glucose-6-phosphate in the blood of the crayfish *Orconectes virilis*. Their observations, however, do not provide a picture of the metabolic changes taking place within the animal. The source of glucosyl oligosaccharides and their metabolic role in the animal is not known. The significance of the mucopolysaccharide in the metabolism of the animal is not understood. Since the material previously determined as glycogen includes this complex polysaccharide as well as glycogen it is of interest to study the variation in concentration of these two polysaccharides in relation to the stages of the intermoult cycle.

The present study throws some light on the normal pattern of carbohydrate metabolism with special reference to the role of glucose as an energy source and a precursor of glycogen, mucopolysaccharide and chitin.

MATERIALS AND METHODS

For the present study both *Hemigrapsus nudus* and *Cancer magister* were used. *Hemigrapsus nudus* was collected at low tide from the vicinity of Charleston, Oregon, and maintained in the laboratory in shallow sea water at temperatures ranging between 10 and 15°C. *Cancer magister* was supplied regularly by Hallmark Fisheries of Charleston and the Oregon Fish Commission Laboratory at Newport, Oregon. The animals were staged by the method followed by Kincaid & Scheer (1952) which is a modification of the method of Drach (1939) and Hiatt (1948).

The total carbohydrates in the blood and the tissue extracts was determined by the anthrone method (Roe, 1955). Glycogen and mucopolysaccharide were also estimated by the same method (Carroll *et al.*, 1956). Blood glucose was estimated by this glucose oxidase method described by Huggett & Nixon (1957). The oligosaccharides were separated by absorption on charcoal (Fishman & Sie, 1958)

and estimated by the anthrone method after elution. The protein-bound serum polysaccharide was estimated by the anthrone method as suggested by Bjornesja (1955).

The cuticle was prepared according to the methods used by Fraenkel & Rudall (1940) for insects. The polysaccharides were precipitated from alkali extracts and separated by paper electrophoresis at pH (Rienits, 1953; Bera *et al.*, 1955) using the Karler-Kirk curtain electrophoresis apparatus. The polysaccharides were stained on the paper by the method of Koiw & Gronwall (1952) and Leitner & Kerby (1954). The sulphate content of the mucopolysaccharide was studied by the method of Hawk *et al.* (1954) and determined by the turbidometric sulphate determination (Zdybek *et al.*, 1960). Isotopic studies using ^{14}C glucose and ^{14}C maltose were conducted as described by Kittinger (1953) and Hu (1958).

Several solvent systems were used for chromatographic separation. For the oligosaccharides the solvent systems described by French *et al.* (1950), Jermyn & Isherwood (1949) and Boyly & Bourne (1953) were used. For monosaccharides, besides the above-mentioned solvent systems, those described by Lindberg *et al.* (1953), Lindberg & Wickberg (1953) and Hough (1954) were also used for nucleotides. The Pabst procedure as described by Hu (1958) was followed. The colour reagents used were those described by Trevelyan *et al.* (1950), Partridge (1949), Wood (1955) and Toennies & Kolb (1951).

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RESULTS

(1) *Blood carbohydrates*

The carbohydrate constituents of the blood of *Hemigrapsus nudus* and *Cancer magister* were examined by paper chromatography. Both the crabs showed the

TABLE 1—CONCENTRATION OF BLOOD CARBOHYDRATES IN *Cancer magister*
Typical values expressed as mg glucose equivalents per 100 ml blood.
All determinations done on the same sample

Substance	Concentration (mg/100 ml)	% of total carbohydrate
Glucose	5·7	12·8
Glucose-6-phosphate	7·2	16·2
Oligosaccharides	10·92	24·5
Glycoproteins	13·2	30
Unidentified fraction	7·98	16·5
Total carbohydrates	44·3	

presence of glucose, glucose-6-phosphate, maltose, maltotriose, maltotetrose and traces of fucose and galactose. No trace of trehalose was found. Besides these a substantial amount of glycoproteins was also observed in the blood.

The typical concentration of the different fractions in the blood of *Cancer magister* is shown in Table 1. The concentration of glucose and glucose-6-phosphate

varied only slightly in specimens collected at different seasons and these account for about 10 per cent and 10–15 per cent of the total carbohydrates respectively. Oligosaccharides showed more variation in concentration but typically accounted for 20–25 per cent of the total carbohydrates of both blood and digestive gland.

TABLE 2—OLIGOSACCHARIDE CONTENT OF *Hemigrapsus nudus* IN RELATION TO DIET

Treatment	Oligosaccharides (mg glucose equivalent/100 g) ± standard deviation of mean
Freshly collected	42·2 ± 3·0
Starved for 10 days	28·6 ± 10·3
Fed on liver for 10 days	68·6 ± 3·0
Fed on rice for 10 days	69·2 ± 4·7
Fed 100 mg glucose (12 hr)	86·1 ± 5·1

Feeding the animals with carbohydrate-rich food and also with glucose itself had a great influence on the oligosaccharide content. *Hemigrapsus nudus* was fed on liver, rice and glucose solution (glucose solution was introduced into the stomach by polythylene tubing). Table 2 summarizes the result of these experiments. The oligosaccharides increased after feeding carbohydrate-rich food, indicating that the dietary carbohydrates are a source of blood oligosaccharides.

TABLE 3—BLOOD SUGARS IN *Cancer magister* AFTER INTRODUCING GLUCOSE INTO THE STOMACH

Concentrations in mg per 100 ml

Time after introducing glucose (hr)	Total carbohydrates	Glucose	Oligo-saccharides	Other carbohydrate fractions
0	36·66	2·6	7·21	26·9
3	52·08	4·85	20·30	26·0
6	52·60	4·62	21·12	26·9
9	68·95	5·35	40·01	23·6
12	78·54	6·0	45·99	26·5
24	56·25	3·35	26·21	25·6
48	48·22	3·11	18·0	27·1
72	34·1	2·5	6·82	24·78

In *Cancer magister* no feeding experiment could be done as the animal refused to eat in captivity. However, glucose was introduced through tubes into the stomach and the blood oligosaccharides estimated at different intervals. Table 3 gives the result of the experiment. The blood oligosaccharides increased after feeding glucose and returned to the initial level only after 3–4 days.

Injection of glucose directly into the blood of *Cancer magister* showed a slightly different result. The level of different carbohydrate fractions was estimated for several hours after injection of glucose. Table 4 summarizes the result. When glucose is absorbed from the alimentary canal, the only fraction that increases in

TABLE 4—BLOOD SUGARS IN BLOOD OF *Cancer magister* AFTER INJECTION OF 50 mg GLUCOSE

Time after injection of glucose (hr)	Carbohydrates expressed as mg/100 ml blood			
	Total carbohydrate	Glucose	Oligo-saccharide	Residual
0	32·2	2·72	7·2	22·3
3	48·82	13·91	11·11	23·8
6	60·62	15·20	16·99	28·5
9	63·28	14·61	17·28	40·9
12	61·10	11·92	18·01	31·2
24	52·01	8·22	15·11	28·7

the blood is the oligosaccharide fraction, whereas if glucose is injected into the blood there is a sharp but relatively brief increase in a fraction other than glucose and oligosaccharides. The oligosaccharides showed a smaller but longer lasting increase. Repetition of this experiment showed that the fraction which increases initially is the one containing glucose-6-phosphate.

TABLE 5—RADIOACTIVITY IN BLOOD SUGARS AND HEPATOPANCREAS POLYSACCHARIDES AFTER ^{14}C GLUCOSE INJECTION INTO *Cancer magister*

Specific activity, counts per minute per mg

Fraction	Time after injection (hr)				
	3	6	9	12	24
Blood glucose	21	18·5	11	9	7
Blood glucose-6-phosphate	16·2	15·1	15·6	7·2	Trace
Blood maltose	14	11·5	16	17	13
Blood maltotriose	—	4·5	13	17	12
Blood maltotetrose	—	Trace	13	16	12·5
Hepatopancreas glycogen	—	—	—	—	20
Hepatopancreas mucopolysaccharide	—	—	Trace	Trace	17·8

(2) *Experiments with injection of labeled glucose and maltose*

Following injection of ^{14}C glucose in *Cancer magister* the different fractions of blood carbohydrates, glucose-6-phosphate, maltose, maltotriose and maltotetrose

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showed radioactivity. The appearance of radioactivity in the different fractions in relation to the time after injection of the labeled materials is shown.

It is clearly indicated that glucose is a precursor of glucose-6-phosphate and oligosaccharides. Radioactivity appeared as early as 2 hr in glucose-6-phosphate and maltose (not shown in the table) but only after 6–9 hr in maltotriose and maltotetrose, with a maximum in all these at about 12 hr and a slow decline in activity in all the fractions thereafter. The liver polysaccharides showed detectable amounts of radioactivity only at 24 hr at which time the activity of the oligosaccharides had begun to decline.

Labeled maltose was also injected and the blood was analysed for radioactivity in the carbohydrate fractions. Maltotriose and maltotetrose showed activity but no detectable amount of radioactivity appeared in the glucose fraction.

(3) *Glycoproteins in the blood*

Glucose, glucose-6-phosphate and oligosaccharides together could not account for all the carbohydrates present in the blood. The glycoprotein fraction showed great variation; 20–40 per cent of the total carbohydrates could be accounted for as acid-soluble glycoproteins. Injection of glucose did not cause any considerable change in this fraction nor could radioactivity be detected in this fraction after labeled glucose and maltose injection. The glycoprotein on hydrolysis yielded glucose and glucosamine.

(4) *Glycogen*

Glycogen is stored in large amounts in the hepatopancreas and shows great variation in its quantitative distribution. In *Cancer magister* the hepatopancreas contains 0·3–1·2 g of glycogen per 100 g weight of the tissue. Both feeding and injection of glucose caused increase in glycogen when estimated after 48 hr. In *Hemigrapsus nudus* also feeding and injection of glucose produced the same result, as shown in Table 6.

TABLE 6—GLYCOGEN CONTENT IN MALE *Hemigrapsus nudus*

Treatment	No. of animals studied	Mean wet wt. (g)	Glycogen (mg/g) ± standard deviation	Glycogen per animal	Glucose recovered (%)
Fresh from the field	5	8·16	7·6 ± 0·098	62	—
100 mg glucose fed	6	9·02	10·57 ± 0·009	95	33
50 mg glucose injected	6	9·43	10·46 ± 0·114	98	72

Injections of radioactive glucose and maltose were followed by appearance of radioactivity in the glycogen fraction, indicative that glucose and maltose are precursors of glycogen; ^{14}C glucose appeared in detectable amounts in the glycogen fraction by 24 hr whereas maltose appeared within 12 hr. This suggests that maltose is an intermediate between glucose and glycogen.

(5) *Mucopolysaccharide*

The presence of an acid mucopolysaccharide in the hepatopancreas and cuticle in *Hemigrapsus nudus* and its quantitative distribution in normal C_4 stage animals was previously reported by Meenakshi & Scheer (1959). In labeled glucose injection experiments the glucose moiety of the polysaccharide alone showed labelling and the fucose and galactose moieties did not show any trace of radioactivity, confirming the previous results. The presence of the polysaccharide in the cuticle shows interesting relations to the stages of moult cycle. In the newly moulted animals (moulted in the laboratory) there were only traces of the polysaccharide in the cuticle. In animals of Stage B the cuticle showed a higher

TABLE 7—RADIOACTIVITY IN CHITIN AFTER INJECTION OF ^{14}C GLUCOSE INTO *Hemigrapsus nudus*

Stage	Time after injection (hr)	Specific activity of chitin (c.p.m./mg)	% of total injected radio activity recovered in chitin
A	24	21	7·8
A	48	27·5	14·83
B_1	48	19·2	15·28
B_2	48	14	10·72
C_1	48	1·5	0·71
C_2	48	—	—
C_3	48	—	—
C_4	48	—	—
D_1	48	—	—
D_2	48	—	—
D_3	24	—	Trace
D_4	24	—	4·3

amount of acid mucopolysaccharide than Stage A. When ^{14}C glucose was injected into Stage A and Stage B animals the mucopolysaccharide of the cuticle showed radioactivity whereas in Stage C and D animals no radioactivity appeared in the mucopolysaccharide of the cuticle. This indicates that the mucopolysaccharide is deposited in the cuticular region solely during the postmoult stages. The mucopolysaccharide formed during the intermoult and premoult stages is stored in the hepatopancreas, presumably to be used for the next moulting season.

On analysis (Hawk *et al.*, 1954) the polysaccharide was found to be sulphated. Quantitative analysis showed that there is 2–2·4 mg of sulphur per 100 mg of glucose present in the mucopolysaccharide.

(6) *Chitin*

The incorporation of labeled glucose into chitin was examined at different stages of the intermoult cycle in *Hemigrapsus nudus*. ^{14}C glucose was injected into the animal and chitin isolated after 24 or 48 hr. The results are summarized

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in Table 7. Chitin is actively synthesized in postmoult stages and never in the intermoult or premoult stages except for traces in early C and traces in late D_4 stages.

(7) *Radioactivity in the respiratory* CO_2 *after injection of labeled maltose*

The presence of radioactivity in the respiratory CO_2 after ^{14}C glucose injection was observed by Hu (1958) and later by Bergreen *et al.* (1961). The appearance of radioactivity after ^{14}C maltose injection was studied under different experimental conditions and the results obtained are tabulated in Table 8.

TABLE 8—APPEARANCE OF ^{14}C IN RESPIRATORY CO_2 AFTER INJECTION OF LABELED MALTOSE WITH AND WITHOUT GLUCOSE

Collection period (hr)	Total radioactivity in the respiratory CO_2 (c.p.m.) after injection of		
	0·2 μc maltose	2 μc maltose + 10 mg glucose	0·2 μc maltose + 10 mg glucose every 24 hr
1–12	1120	Trace	Trace
13–24	1800	1220	340
25–36	1016	1624	Trace
37–48	600	840	Trace
49–60	Trace	680	Trace
61–72	Nil	Trace	Trace

When ^{14}C maltose alone is given, large amounts of radioactivity are observed in the respired CO_2 within the first 2 hr, whereas when 10 mg of ordinary glucose is administered along with ^{14}C maltose only traces of radioactivity appear in the first 24 hr and none later. When 10 mg of glucose is given every 24 hr only traces of radioactivity appeared in the respired CO_2 throughout the experiment.

DISCUSSION

A probable pathway of glucose metabolism in crustaceans is presented in Fig. 1. This is based on evidence given above and inferences drawn from known mechanisms of carbohydrate metabolism in other organisms. Though the enzyme hexokinase is not so far demonstrated in the crustaceans, the presence of glucose-6-phosphate in crabs and the fact that it is formed from glucose suggests that glucose enters crustacean metabolism by a hexokinase-catalysed transphosphorylation. The presence of glucose-6-phosphate in the blood is of great interest. This indicates the presence of an "extracellular glycolysis" system in crustaceans which liberates phosphorylated intermediates, as observed by Shaw & Stadie (1959) in rat diaphragm muscle. The phosphoglucomutase reaction and glucose-1-phosphatase have not been demonstrated in crustaceans. The process is inferred because of their universal occurrence.

The observations in crabs clearly establish this synthesis of maltose from glucose, though the role of UDPG in this system is drawn from analogy with other systems (Hassid *et al.*, 1959; Strominger, 1960). Carbon of labeled glucose appears in the nucleotide fraction after injection (Hu, 1958). The synthesis of glycogen probably involves UDP glucose–glycogen transglycosylase rather than phosphorylase as

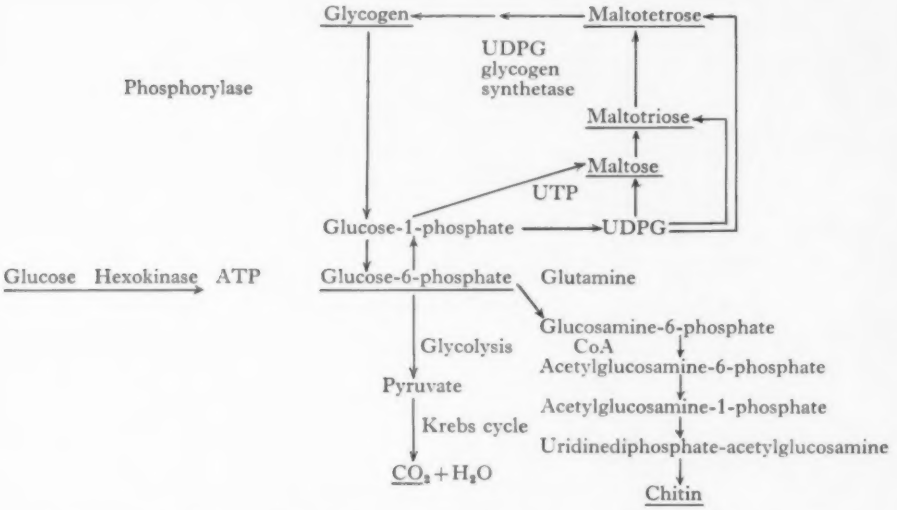

FIG. 1. Probable pathways of glucose metabolism in crustaceans. ATP = adenosine-triphosphate; CoA = Coenzyme A; UTP = uridinetriphosphate; UDPG = Uridine-diphosphoglucose. Underlined substances are labeled after administration of ^{14}C labeled glucose.

has recently been reported for mammals (Schmidt *et al.*, 1959) and insects (Trivelloni, 1960). The enzyme phosphorylase has been studied in lobster muscles by Cowgill (1959a, b) but the relative stability of glycogen in crustacean tissue (Renaud, 1949; Neiland & Scheer, 1953; Scheer & Scheer, 1951) indicates that the phosphorylase activity is relatively low. Our observations suggest that maltose oligosaccharides are intermediates in glycogen synthesis. Since maltose carbon does not appear in glucose, maltose is not present in crabs and synthesis of oligosaccharides by an amylomaltase as reported in plants (Hehre, 1951) seems unlikely. The oxidation of maltose must be taking place through glycogen synthesis, phosphorolysis, glycolysis and oxidation. This view is supported by the fact that glucose spares maltose utilization. In the absence of alimentary carbohydrate the blood glucose probably originates from phosphorolysis followed by hydrolysis of glucose-1-phosphate. There is no report of the enzyme hexose-1-phosphatase in crustaceans though it has been reported in silkworm blood (Faulkner, 1955). If the above inferences are correct it appears that the major pathway for glycogenesis

in crustaceans is not merely a reversal of, but is rather distinct from that for glycogenolysis. There is good reason to believe that the same distinction may occur in mammalian tissue (Stetten & Stetten, 1960; Sie & Fishman, 1958).

The presence of sulphated mucopolysaccharide is of interest. Its probable significance in calcification was discussed in a previous paper (Meenakshi & Scheer, 1959). The fact that this substance is deposited in Stages A and B in the cuticle and that calcification of the cuticle takes place in these stages and is completed in the C stages also indicates that this substance must be important in calcification.

Little is known of the synthesis of chitin in crustaceans in spite of the relatively unique position occupied by this polysaccharide. In insects synthesis of chitin occurs to a limited extent in the premoult period but most of the chitin is synthesized in the period after moulting (Dennel & Malek, 1956). In the crab *Hemigrapsus nudus* the major portion of the required chitin is synthesized in the postmoult period. The pathway of chitin synthesis indicated here is inferred from studies of Glaser & Brown (1957a, b) on fungi. Obviously these studies should be extended to arthropods, which must be studied during the postmoult period. Carey and Wyatt (1960) have recently reported the occurrence of uridine diphosphate acetylglucosamine in insect pupae.

It is now clear that glucose is oxidized by crustaceans (Hu, 1958; Bergreen *et al.*, 1961); our experiments with maltose show that this carbohydrate is also oxidized, and from our other results we must conclude that glycogen formation followed by glycolysis is the most probable pathway for this oxidation.

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SPONTANEOUS ACTIVITY IN THE ISOLATED NERVES OF PULMONATE MOLLUSCS

C. J. DUNCAN

Department of Zoology, University of Liverpool

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Abstract—1. Spontaneous activity in isolated pieces of the pallial nerves of a variety of pulmonates is described and it is suggested that it originated from ganglion cells along their length.

2. The active units show little response to changes in the concentration or in the osmotic pressure of the bathing fluid.

3. Cation imbalance of the bathing solution produced marked changes in the frequency of the activity, monovalent and divalent cations having opposite effects. Thus, potassium ions are excitatory in excess, while their absence reduces the rate of firing.

4. The action of pharmacological agents suggests that many of the active elements are cholinergic, being stimulated by acetylcholine, while anticholinesterases inhibit activity. Curare, however, was without demonstrable effect at relatively high concentrations.

INTRODUCTION

SPONTANEOUS activity has been described in a variety of simple preparations, probably the best documented being that of the lobster heart ganglion (see Maynard, 1953; Terzuolo & Bullock, 1958). The purpose of this paper is to give a preliminary account of the apparently spontaneous activity in isolated pieces of nerves of pulmonate molluscs, and the effect of altering the ionic composition of the bathing fluid and of the addition of various drugs and chemical mediators. The preparation is very convenient for such an investigation since it is so simple and especially because in many of the nerves the activity of individual units can be distinguished.

The occurrence of spontaneous activity in the peripheral nerves of the giant African snail *Archachatina marginata* has been mentioned by Nisbet (1956). Nervous activity in the central nervous system of the slug *Agriolimax reticulatus* has been described by Hughes & Kerkut (1956), and it has been shown that the isolated pedal ganglion is sensitive to changes of osmotic pressure (Kerkut & Taylor, 1956). Kerkut (1959) has extended these observations by tentatively suggesting that the increased activity of hydrated slugs may be the direct consequence of the increase in activity of the spontaneously active cells in the pedal ganglion, following dilution of the medium in which they are bathed. The organization of the central nervous system and the effect of temperature on conduction and ganglionic transmission in the slug *Ariolimax columbianus* have been described in a series of papers by Turner (1951, 1953) and by Turner & Nevius (1951).

During this investigation spontaneous activity has been found in isolated pieces of nerve of both stylommatophoran and basommatophoran pulmonates, but most of the experiments were carried out using the pond snail *Lymnaea stagnalis* (L.), a species which is common in England in ponds and canals where the water is hard and there is an abundance of vegetation. The morphology of the nervous system of *L. stagnalis appressa* has been described by Carriker (1946) and his terminology for the nerves has been followed (see Fig. 1).

MATERIALS AND METHODS

Specimens of *Lymnaea stagnalis*, *Planorbarius corneus* and *Physa* spp. were collected from local ponds or canals and maintained in laboratory aquaria. *Helix pomatia* and *H. aspersa* were obtained from laboratory dealers and were kept on slightly damp peat moss and fed on cabbage during the short period before the experiments. After dissecting the snails in an appropriate bathing solution, recordings were made first from the intact nerve, and then after cutting peripherally or, more usually, both centrally and peripherally. The isolated nerve was then transferred to a separate dish of bathing fluid. A number of physiological salt solutions have been developed for work with *Lymnaea* and in the present investigation the composition of the bathing fluid used for the freshwater basommatophorans, as in previous studies (Duncan, 1958), followed that given by Pantin (1948, p. 67) and proved satisfactory. Other physiological solutions for *Lymnaea* have been used by Carriker (1946) and Fraser (1946), and by Horstmann (1958).

The isolated piece of nerve was looped over external, silver/silver chloride electrodes and then raised into liquid paraffin or into the air. The nerve could be briefly lowered into other solutions, which either differed in ionic composition or contained pharmacological agents, and their effect observed. Temperature was maintained at $19 \pm 1°C$. Conventional, a.c.-coupled, differential amplifiers were used and EF86 valves proved suitable, the smallest test signal which could be distinguished being 5 μV. The amplified impulses were led to a Cossor double-beam oscilloscope and photographed on high-speed recording paper.

NERVOUS SYSTEM OF *LYMNAEA STAGNALIS*

Fig. 1 shows diagrammatically the arrangement of the ganglia of the central nervous system of *Lymnaea stagnalis* and also the nerves leaving the parietal and abdominal ganglia. The nerves principally studied were the anterior left and right, and median pallial nerves and the splanchnic nerve. Since bipolar and multipolar ganglion cells have been described histologically in the intestinal nerve of *Helix* (Schlote, 1955, 1957) and in the nerve bundles of the clam *Mya arenaria* (Horridge, 1958), it seemed probable that comparable cells would be present in the nerves of *Lymnaea*, and responsible for at least some of the spontaneous nerve activity. Such ganglion cells were found to be present in the pallial nerves examined and are similar to those described in *Helix*. Their nuclei are large (about 45 μ diameter) and stain deeply with iron haematoxylin.

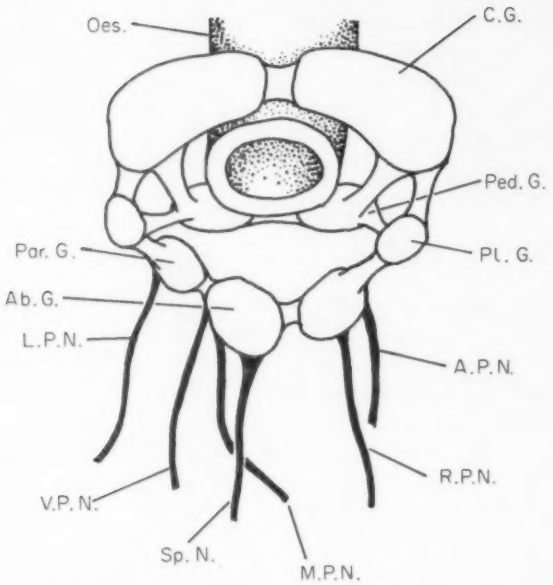

FIG. 1. Diagram of a dorsal view of the central nervous system of *L. stagnalis*, showing the origin of the nerves studied. After Carriker (1946). A.P.N., anterior pallial nerve; Ab.G., abdominal ganglion; C.G., cerebral ganglion; L.P.N., left pallial nerve; M.P.N., median pallial nerve; Oes., oesophagus; Par.G., parietal ganglion; Ped.G., pedal ganglion; Pl.G., pleural ganglion; R.P.N., right pallial nerve; Sp.N., splanchnic nerve; V.P.N., ventropallial nerve.

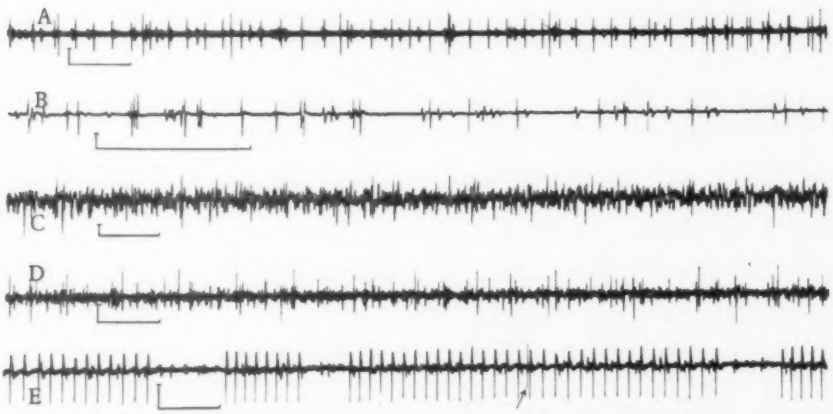

FIG. 2. Spontaneous activity in isolated pieces of nerves of *L. stagnalis*. Horizontal scale = 1 sec; vertical scale = test pulse for each record. A, Left pallial nerve (100 μV). B, Same preparation after an interval of 5 min and at a faster camera speed (100 μV). C, Right pallial nerve (25 μV). D, Splanchnic nerve (45 μV). E, Anterior pallial nerve. The action potential from a different unit is indicated by an arrow (200 μV).

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(a) *Spontaneous discharge*

The pattern of activity typically observed in three different nerves, the left and right pallial and the splanchnic, is shown in Fig. 2. In general there is a similarity between individual snails in the activity of each nerve. Thus, the left pallial nerve (Fig. 2 A and B) is particularly suitable for identifying the different active elements, the total activity being about 11 per second. Spike amplitudes, as recorded, range from 10 to 140 μV, although, as will be shown later, units generating larger potentials are also present and can be stimulated into activity, but are usually quiescent in the undisturbed preparation. Analysis of records from this nerve over longer periods of time suggest that each active unit maintains its own rate of firing with short intervals of increased or reduced activity. In the splanchnic and right pallial nerve there is much activity from units producing smaller action potentials (Fig. 2 C and D).

Occasionally, a preparation was obtained which generated large spike potentials which discharged regularly to give a rhythmical rate of firing. Examples are shown in Fig. 2 E and also in Fig. 4 C (*Helix*). Such rhythmic activity frequently continued for 20 min, usually in bursts of varying lengths separated by brief quiescent periods. Fig. 2 E of the median pallial nerve illustrates a unit firing with potentials of characteristic size and shape in bursts lasting 1–6 sec and at a frequency of about 5 per sec. The bursts are of varying duration and the presence of one spike potential produced by a different unit is indicated by an arrow.

When the nerve is cut centrally, there is a massive discharge which falls gradually and after 5 min the isolated piece of nerve settles to a steady rate of firing (Fig. 2 A) and, provided the preparation is kept in saline solution at about 7°C, maintains this activity for 24 hr, although a slight decrease in frequency can be detected after 6 hr. If the preparation was exposed to the air for 3 or more minutes the nerve dried and the frequency of the activity was reduced. Hughes & Kerkut (1956) noted a similar effect in their studies on the pedal ganglion of *Agriolimax*. No counts of nerve activity were therefore made on preparations exposed to the air. This technique was used only when the effect of ions or pharmacological agents was being studied, and when the result was immediate and striking. Control experiments, lifting the piece of nerve in and out of the bathing fluid, showed little change in the pattern of activity.

(b) *The effect of varying the concentration of the bathing fluid*

Hughes & Kerkut (1956) have shown that activity in the isolated pedal ganglion of the slug *Agriolimax* is affected by variations in the concentrations of the bathing solution, dilution increasing the activity. The ganglion responds to changes in the osmotic pressure of the solution and not to its ionic concentration (Kerkut & Taylor, 1956). These workers suggest that certain cells in the ganglion act as osmoreceptors. The standard bathing solution which they used was 0·7 Locke and a change from this concentration to 0·5 Locke produced four times the frequency of activity.

Responses of pieces of nerves of *L. stagnalis* were much less dramatic when immersed in solutions of different concentrations. Counts lasting 20 sec were

made at intervals. On dilution of the bathing solution to one-fifth normal concentration, a reduction in the rate of activity was noted after 15 min, and a one-tenth standard solution eliminated spike potentials in 3 min, although apparently normal activity was regained when the preparation was returned to the original bathing solution. Doubling the concentration gave the immediate response of increasing the frequency of activity four times, but this fell during the following 90 sec, and thereafter was maintained at about 25 per cent above the normal level. However, these are responses to abnormally great changes in the composition of the bathing fluid. Raising the osmotic pressure of the bathing fluid equivalent to twice normal by the addition of glucose produced little significant change in the rate of activity.

(c) *The effect of cations*

The isolated nerve preparation is, however, sensitive to changes in the cation composition of the bathing medium. Such a sensitivity has been reported in other active units; e.g. the heart ganglion of *Limulus* (Prosser, 1943) and the ganglionic

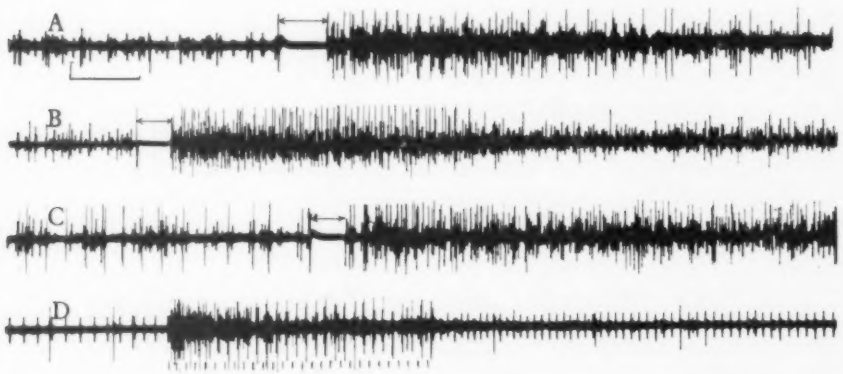

FIG. 3. Activity in nerves of *L. stagnalis*. Period of submergence in test fluids is indicated by an arrow. Vertical scale = 10 μV; horizontal scale = 1 sec. A, Isolated splanchnic nerve, response to $5 \times K^+$. B, Isolated splanchnic nerve, response to acetylcholine, 0·05 mg/ml. C, Isolated splanchnic nerve, initial excitatory response to nicotine (10^{-3} M). D, Activity in the left pallial nerve following repetitive stimulation of the right pallial nerve. Time of stimuli shown below the trace; for further explanation see text.

trunk of the heart of the lobster, *Panulirus* (Matsui, 1957). As in these other preparations, the monovalent and divalent cations had opposite effects on the activity of isolated pieces of the nerves of *Lymnaea*. The bathing solutions were prepared by raising either the KCl, $CaCl_2$ or $MgCl_2$ three or five times above normal. The response of individual units varied, but a five-fold increase in potassium generally produced an immediate increase in total activity (Fig. 3 A), whilst bathing solutions lacking potassium inhibited the majority of active units

and reduced the frequency of the remainder. When treated with excess potassium, some units stopped firing but the majority increased in frequency and others, generating large, fast, spike potentials, which are usually quiescent in normal preparations, were stimulated into activity.

By touching the damp preparation in air with a fine, earthed, silver wire on which a thin film of sodium chloride had been allowed to evaporate to dryness, it proved possible to stimulate differentially the rhythmical discharge of only one of these normally quiescent units. Excess calcium or magnesium (three or five times above normal) produced an immediate and marked fall in activity, whilst bathing solutions lacking either of these ions caused a small increase in the total rate of firing. The effect of any of these ions was reversed when the preparation was returned to the normal bathing fluid.

(d) *The effect of neurotropic substances*

Ramsay (1940) found that the retractor muscle of the buccal mass of *Helix pomatia* is sensitive to acetylcholine, responding with a contraction to a concentration of 0·0012 mg/ml, and that eserine modifies neuromuscular transmission. The experiments concerning the effect of acetylcholine on molluscan hearts has been reviewed by Krijgsman (1955) and the evidence suggests that they are supplied by inhibitory fibres which have cholinergic properties. The effect of a number of neurotropic substances on isolated pieces of nerve of *L. stagnalis* was tested. The substance was dissolved in the bathing fluid and then the preparation was briefly submerged. The results can be summarized as follows:

Acetylcholine in concentrations of 0·02–0·05 mg/ml produced an immediate increase in activity (Fig. 3 B). Additional units are stimulated into activity and the rate of firing of all units is greatly increased. After about 5 sec the initial frequency falls but supranormal activity is maintained for at least 1 min. These responses are comparable with the discharge initiated in mammalian post-ganglionic fibres when the ganglion is perfused with a Ringer solution containing 0·1 mg/ml acetylcholine (see Bronk, 1939). Concentrations of 0·1 mg/ml and above completely suppress activity in the snail preparation, but the effect is reversible and the spike potentials return to normal when replaced in standard bathing fluid. Solutions of 0·005 mg/ml have no significant effect.

Eserine destroys or inactivates cholinesterase in vertebrate preparations. Concentrations of 0·2 mg/ml (5×10^{-4} M) produce an immediate reduction of activity in the snail preparation, but the effect is reversible.

Di-isopropylfluorophosphate (D.F.P.), an anticholinesterase, causes a complete and irreversible cessation of activity after 30 sec at concentrations of 10^{-3} M.

D-tubocurarine at the relatively high concentration of 1 mg/ml had little significant effect on the preparation after 2 hr. In some experiments the two parietal ganglia and the abdominal ganglion were isolated together with the left and right pallial nerves which serve them (see Fig. 1). Both nerves were cut peripherally. Stimuli were applied to the right pallial nerve and the resulting spike potentials recorded in the left pallial nerve. Immersion for 2 hr in a fluid

containing 1 mg/ml curare failed to block synaptic transmission via these ganglia. Ramsay (1940) records that curare failed to block neuromuscular transmission in the retractor muscle of the buccal mass of *Helix*.

Nicotine (Fig. 3 C), at a concentration of 10^{-2} M, has an initial excitatory effect, but after 1 min it is followed by a marked, reversible decrease in activity below the initial level. Ganglionic transmission in the abdominal and parietal ganglia, in the preparation described above, is blocked by a 6×10^{-3} M nicotine solution, but not at a concentration of 6×10^{-4} M. Turner & Nevius (1951) similarly found that nicotine (10^{-4} M to 10^{-3} M) blocked synaptic transmission in the ganglia of a slug.

ACTIVITY IN OTHER SPECIES

Spontaneous activity was also found in isolated pieces of nerves of other pulmonates, namely, *Planorbarius corneus*, *Physa* spp., (Basommatophora); *Helix pomatia* and *H. aspersa* (Stylommatophora). Examples are illustrated in Fig. 4.

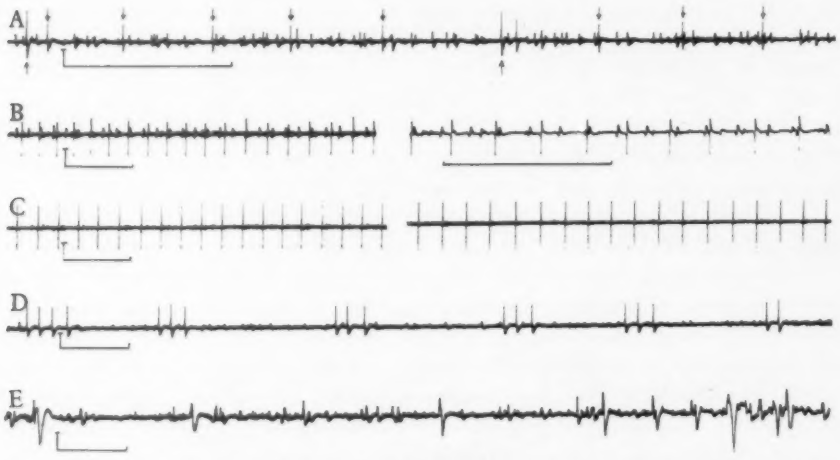

FIG. 4. Spontaneous activity in isolated pieces of nerves of pulmonates. Horizontal scale = 1 sec, except in record D; vertical scale = individual test pulse. A, *Physa* sp., median pallial nerve. Activity of two units indicated by arrow (400 μV). B, *Planorbarius corneus*, right pallial nerve. The second record, at a faster camera speed, follows after an interval of 30 sec (400 μV). C, *Helix pomatia*, right mantle nerve; second record follows after an interval of 10 min (400 μV). D, *Helix aspersa*, nerve to anterior portion of the hermaphrodite duct. Horizontal scale = 0·4 sec (100 μV). E, *H. aspersa*, one of the main pedal nerves (250 μV).

Fig. 4 A shows typical activity in a pallial nerve of *Physa* sp., in which the spike potentials of two of the units are identified by arrows above and below the base line. Rhythmical discharge of one characteristic unit amongst the total activity in the right pallial nerve of *Planorbarius corneus* is seen in Fig. 4 B. The second half of this record, at a faster camera speed, follows after an interval of

5 min. Preparations containing only one active generating large unit spike potential are particularly readily obtained from *Helix* spp. Fig. 4 C illustrates such a case in an isolated piece of nerve supplying the right mantle of *H. pomatia*. The second part of the record follows the first after an interval of 15 min and shows that the rhythmic activity has changed only very slightly. Fig. 4 D shows a unit in the nerve supplying the anterior part of the hermaphrodite duct of *H. aspersa*. This unit fires in bursts of two to four spike potentials. The activity in one of the main pedal nerves of *H. aspersa* is shown in Fig. 4 E, where large, slow spike potentials were found.

DISCUSSION

Hughes & Kerkut (1956) have suggested that the nervous tissue of pulmonates is particularly suitable for the study of isolated preparations, being normally bathed in a haemocoel. An isolated piece of nerve represents a very artificial preparation, but observations in these experiments have suggested that the patterns of activity found closely resemble those recorded from nerves before isolation. The rhythm, however, can be affected by activity elsewhere in the nervous system and it is hoped to present a more complete picture in a later publication. This is shown by a preparation of the left and right pallial nerves separated by the two parietal ganglia and the abdominal ganglion, both nerves being cut peripherally as described previously. Activity was recorded in the left pallial nerve; two units, firing rhythmically, can be seen (Fig. 3 D). Stimuli applied to the right pallial nerve, at the rate of 7 per second produced compound action potentials in the left pallial nerve, and when the stimuli were stopped the original rhythmic activity continued with one of the units firing at an accelerated rate. This activity originated in the nerve and not from the ganglion cells of the brain, since it continued when the nerve was subsequently isolated.

The role of such spontaneous activity in the living animal is unknown, but the results suggest that it is quite widespread among the higher pulmonates. It is most probable that it is generated by the ganglion cells which were found in the pallial nerve. However, it should be pointed out that the nerves are surrounded by a thick sheath and the electron microscope studies by Schlote (1955, 1957) have revealed the presence of muscle cells in the perineureum of the intestinal nerve of *Helix pomatia*. It is possible that some of the activity observed originated from such muscle cells. The slower potentials recorded from the pedal nerve of *H. aspersa* (Fig. 4 E) might come from such a source. Apart from this, spontaneous activity appeared to be confined to the longer nerves, running posteriorly, which serve the parietal and abdominal ganglia. The sensitivity of the cockroach nerve cord to chemicals was greatly increased when its connective tissue sheath was removed (Twarog & Roeder, 1956), and it may have a similar effect on the pulmonate preparations.

The response of the active units to cation imbalance and to neurotropic substances agrees well with the idea that they are spontaneously active, cholinergic elements, but it is interesting to note that Ramsay (1940) also found that curare

was without demonstrable effect on the neuromuscular junction of the retractor muscle of the buccal mass of *Helix*. The active elements, in contradistinction to the response to cations, are relatively insensitive to overall changes in the concentration of the bathing medium, whereas the pedal ganglion of *Agriolimax* is sensitive to changes of 0·05 Locke solution, i.e. from 0·7 Locke to 0·65 Locke (Hughes & Kerkut, 1956). These observations suggest that sensitivity to osmotic pressure changes is not a general property of pulmonate ganglion cells, and agrees with the conclusions of Kerkut & Taylor (1956) that specialized osmoreceptors are present in the pedal ganglion of the slug.

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ELECTROPHORETIC STUDIES OF BLOOD PROTEINS OF TWO SPECIES OF TOADS AND THEIR NATURAL HYBRID*

W. FOX, H. C. DESSAUER and L. T. MAUMUS†

Departments of Anatomy and Biochemistry, Louisiana State University School of Medicine, New Orleans, Louisiana

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Abstract—1. Hemoglobin and plasma proteins of two species of toads (*Bufo fowleri* and *Bufo valliceps*) and their natural, F_1 hybrid were studied by paper, moving-boundary and starch-gel electrophoresis.

2. Each species possessed a single hemoglobin and a single transferrin. Both hemoglobins and transferrins of the two species migrated at strikingly different rates.

3. The hybrids possessed two hemoglobins and two transferrins, one of each protein from each parent species. The electrophoretic mobilities of the hemoglobins were modified when mixed either artificially or naturally (hybrid hemolysates).

4. The two species possessed several plasma protein fractions in common but a few were specific to each species. Considerable individual variation in concentration of certain plasma fractions was noted.

5. All electrophoretic fractions present in the parent species occurred in the hybrid. With one possible exception, no fraction was found in the hybrid that was not present in one or both of the parent species.

6. Hybrid plasma contained a higher concentration of protein than the plasma of either parental species.

INTRODUCTION

RECENT improvements in physicochemical techniques have made possible highly discriminating analyses of protein mixtures. The resolving power of electrophoresis, and, in particular, electrophoresis through starch-gel, has become so great that a complex protein mixture such as plasma can now be resolved into a great number of apparently homogeneous fractions (Smithies, 1959a). Proteins of such fractions can be identified by rates of electrophoretic migration, staining characteristics, enzyme activity, and binding affinity for such substances as radioactive iron. Combinations of such techniques have been used fruitfully in establishing intraspecific heterology or homology as well as inheritance patterns of mammalian hemoglobins (Huisman, 1958; Allison, 1959), transferrins and haptoglobins (Smithies, 1959a).

There has been little utilization of modern electrophoretic methods in the study of inheritance of blood proteins in hybrids. Brandt *et al.* (1952) found that plasma proteins of hybrids from crosses between chickens and pheasants contained major

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† U.S. Public Health Service, Cardiovascular Summer Fellow.

components with mobilities equal to those present in both parents, other components with mobilities similar to those of one parent and not the other, one component that was present in both parents but obscure in the hybrid and other components present in the hybrid but absent from the parent bird.

In this paper we wish to demonstrate the heterology of hemoglobins and plasma proteins of two species of toads, *Bufo valliceps* and *Bufo fowleri* (*B. woodhousi fowleri*) and to describe the electrophoretic patterns that are inherited by their natural hybrid.

MATERIALS AND METHODS

The distributional ranges of the Gulf Coast toad, *Bufo valliceps*, and of Fowler's toad, *B. fowleri*, overlap in the western Gulf Coast region of the United States. Natural, though sterile, F_1 hybrids of these species may be captured in this area and may be readily identified by means of morphological criteria (Volpe, 1956). Adult specimens of both species and their hybrids were collected along a 5 mile stretch of the east bank of the Mississippi River in Orleans and Jefferson Parishes in Louisiana. Animals were maintained for periods of 1 day to 6 months in plastic terraria and fed meal worm larvae (*Tenebrio molitor*) and ground beef. Findings reported in this paper are based upon analyses of blood of ten hybrids, sixty-five *B. fowleri* and sixty-three *B. valliceps*.

After a fast of 4–7 days, toads to be sacrificed were anesthetized with ether and bled from the heart. Blood was collected in heparinized tubes. Repeated samples were obtained from some animals over a 6 month period by bleeding from a clipped toe directly into a heparinized capillary tube. Blood was centrifuged in the cold and separated from the cells immediately after centrifugation. Aliquots of plasma were subjected to electrophoresis on the same day on which the animals were bled. Excess plasma was stored in a deep freeze for future electrophoretic analyses.

Hemoglobin solutions were prepared as follows: washed red blood cells were suspended in saline and alternately frozen and thawed until the cells were hemolysed. The resulting hemolysate was subjected to high speed centrifugation at 10,000 rev/min to remove cell and nuclear ghosts and insoluble nucleic acid contaminants.

Moving-boundary electrophoresis of dialysed hemoglobins or plasma proteins was carried out in sodium barbital buffer, pH = 8·6, ionic strength, 0·10. Paper electrophoresis was carried out on an LKB unit. Both sodium barbital buffer, pH 8·6, ionic strength 0·05 containing 15 per cent by volume of glycerol and the "high resolution" buffer of Aronsson & Grönwall (1957) were used. The latter has a pH of 8·9, a conductivity of 3·0 mmho and contains a mixture of trihydroxyaminomethane, sodium borate and ethylenediaminetetraacetic acid. Paper strips were stained with either bromphenol blue or with amidoschwartz dyes and the stained strips were scanned in a densitometer. Graphs of optical density against distance migrated were plotted and the relative amount of protein migrating in each peak was estimated from the areas beneath the resulting curves. Total plasma protein

was determined with a biuret technique (Reinhold, 1953). Details of the methods used above may be found in previous papers (Dessauer & Fox, 1956; Ramirez & Dessauer, 1957).

Both horizontal (Smithies, 1955) and vertical (Smithies, 1959b) starch-gel electrophoresis were used to separate hemoglobins and plasma proteins. The borate buffers as recommended by Smithies (1955) were used in all starch-gel separations. Gels were stained with amidoschwartz dye for protein detection. Iron binding protein (transferrin) bands were localized by autoradiography of slabs resulting from electrophoresis of plasma to which radioactive Fe^{59} had been added (Giblett *et al.*, 1959). 0·01 ml of a 0·2 mM solution of ferrous sulfate containing 1 mc of Fe^{59} was mixed with each 0·05 ml of plasma.

RESULTS

Hemoglobins

Hemolysates of red cells of *B. valliceps* and *B. fowleri* and their natural hybrid were readily distinguished upon electrophoretic examination. *B. valliceps* hemoglobin migrated faster in alkaline buffers than *B. fowleri* hemoglobin in either

Table 1—Mobilities of toad hemoglobins in moving-boundary electrophoresis

	No. of animals	Mobility ($cm^2/V/sec$)
Bufo valliceps	4	$-4{\cdot}03 \times 10^{-5}$
Hybrid	1	$-3{\cdot}85 \times 10^{-5}$
Bufo fowleri	4	$-3{\cdot}75 \times 10^{-5}$
B. val. + *B. fowl.* (1:1 mixture)	2	$-3{\cdot}90 \times 10^{-5}$

moving-boundary or zone-electrophoresis (Fig. 1, Table 1). On starch-gel the hemolysate of the hybrid red cells resolved clearly into an intense band that migrated slightly faster than *B. fowleri* hemoglobin and into a second, weaker band that migrated a little slower than *B. valliceps* hemoglobin. The pattern of the hybrid hemoglobins obtained by means of paper electrophoresis was similar except that the resolution into two bands was not clear. We have been unable to resolve the hemolysate of the hybrid into more than a single boundary during moving-boundary electrophoresis. However, as the boundary moved out of the optical path of the cell, its non-gaussian shape was indicative of protein heterogeneity. Since the electrophoretic mobility of this boundary was intermediate between the mobilities of hemoglobins of the parent species, it would appear that protein interaction had altered the mobilities of the two hemoglobins inherited by the hybrid. To test this hypothesis mixtures of hemoglobins from the parent species were subjected to electrophoresis. The artificial mixtures behaved in the same manner as the hybrid hemoglobins. With paper electrophoresis the mixture resolved poorly, each hemoglobin having a mobility different when tested in the

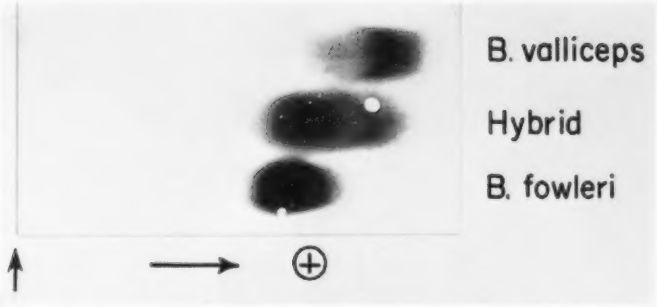

FIG. 1. Starch-gel separation of toad hemoglobins. Vertical arrow indicates position of sample application.

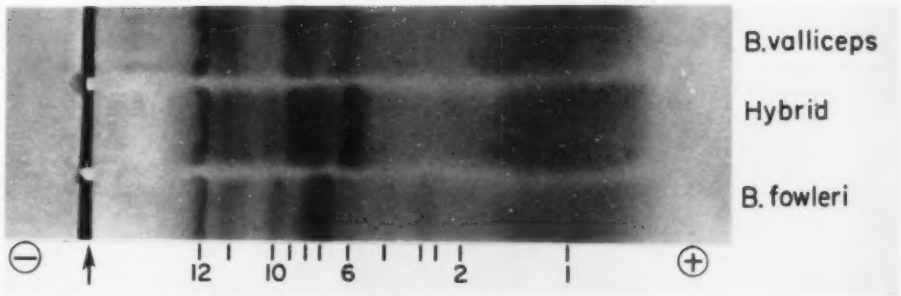

FIG. 3. Starch-gel separation of toad plasma proteins. Vertical arrow indicates point of sample application.

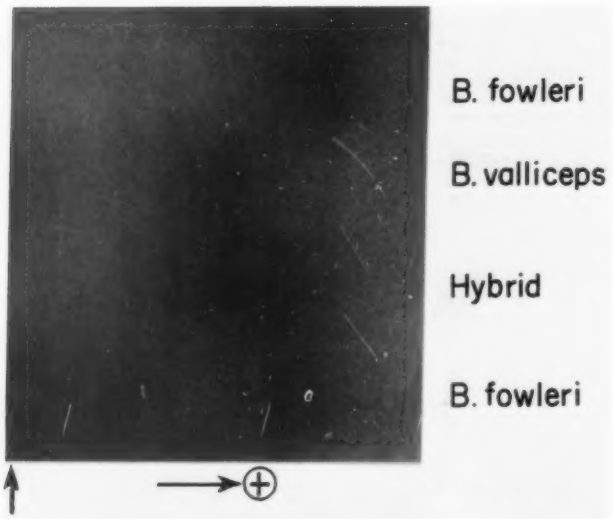

FIG. 6. Autoradiographs of toad transferrins. Diffuse exposures on the anodal side of original positions of sample application (vertical arrow) are due to Fe^{59} borate and do not represent iron bound to protein.

mixture than when tested alone. Similarly, in moving-boundary electrophoresis the pattern of the mixture appeared as a skewed peak that migrated with an intermediate mobility.

Plasma proteins

When subjected to paper electrophoresis in barbital buffer, plasma proteins of these toads resolved into four fractions. Vague differences were noted between

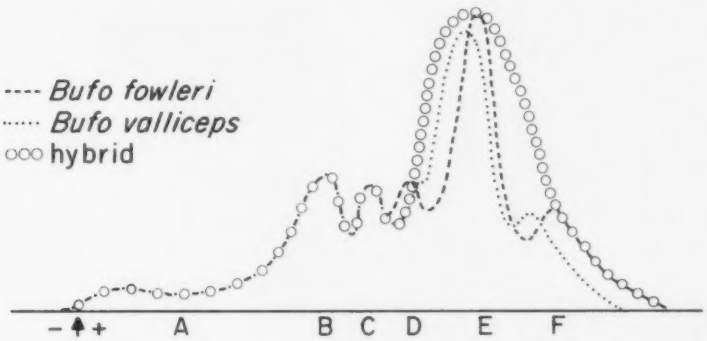

FIG. 2. Paper-electrophoretic patterns of toad plasma proteins. Profiles of the three forms are superimposed to emphasize differences.

patterns of these three forms. The lead anodal fraction of patterns of *B. valliceps* migrated slightly slower than that of *B. fowleri*. This fraction and that of next fastest mobility were more clearly separated from each other in patterns of *B. fowleri* than of *B. valliceps*. Protein of comparable mobilities in patterns of the hybrids migrated as a single, broad, complex fraction.

TABLE 2—CONCENTRATION AND ELECTROPHORETIC DISTRIBUTION OF PLASMA PROTEINS

	No. of animals	Total protein (g/100 ml)	No. of animals	Electrophoretic fractions‡ (per cent)					
				A	B	C	D	E	F
Bufo fowleri	21	2·54 ± 0·87* (0·8–3·2)†	19	10·8 ± 5·5	12·1 ± 4·3	12·2 ± 2·6	10·0 ± 2·0	39·1 ± 4·5	15·8 ± 4·2
Bufo valliceps	27	2·87 ± 0·52 (2·0–3·5)	30	8·9 ± 4·8	12·3 ± 3·1	12·0 ± 2·8	46·4 ± 7·7		20·4 ± 5·2
Hybrid	8	3·70 ± 1·20 (1·6–5·3)	6	8·2 ± 1·4	12·0 ± 2·5	9·5 ± 2·1	47·0 ± 3·0		21·9 ± 3·9

* Mean ± Standard deviation. † Range. ‡ "High-resolution" buffer.

Much clearer resolution of plasma proteins was achieved by use of high resolution buffer. Plasma proteins of both *B. valliceps* and *B. fowleri* resolved into five fractions in this buffer (Fig. 2, Table 2). Fraction D of *B. fowleri* separated completely from adjacent fractions C and E. In contrast fraction D of *B. valliceps*

generally occurred as a ridge on the cathodal side of fraction E. Fractions D, E and F of the two species differed in relative mobilities; the most obvious difference was seen in fraction F, which migrated more slowly in *B. valliceps* than in *B. fowleri*. Proteins of the hybrid in the area of fractions D, E and F were poorly resolved. Rather than three distinct fractions, this area in most patterns appeared as a single broad peak. Fractions B and C were of similar mobility and quantity in the two species of toads and their hybrid.

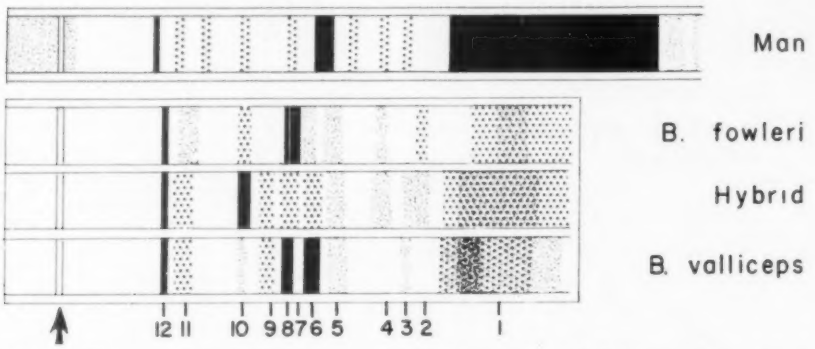

Fig. 4. Diagrams of most frequent starch-gel patterns of toad plasma proteins. Arrow indicates point of sample application. The relative concentration of the protein bands is represented by the density of the vertical bars.

The protein concentration of the plasma of *B. valliceps* and of *B. fowleri* was less than 3 g/100 ml, values typically found in amphibians (Engle & Woods, 1960). Protein of the hybrid, however, averaged 3·70 g/100 ml, 25–50 per cent greater than that of either parent species (Table 2). Six of eight hybrid plasmas analysed contained more protein than plasma from any toad of the parental species. The hybrid with total protein of 1·55 g/100 ml was probably starved in the laboratory. The increased protein of the hybrid migrated chiefly in fractions D, E and F (Fig. 2, Table 2).

Superior resolution of plasma proteins was achieved with starch-gel electrophoresis. Vertical starch-gel patterns of plasma proteins of each of the three toads, separated on adjacent positions, appear in Fig. 3. For descriptive purposes the most common patterns found are identified in Fig. 4.

A number of separations of toad plasma proteins and human plasma proteins were carried out on adjacent positions of the same gel in order to compare the mobilities of toad proteins with those bands found in human plasma (Fig. 4). Band 1 migrated with the cathodal limb of human albumin; bands 2–5 migrated in the area of the post-albumins; bands 6–12 moved in the area between transferrin C and macroglobulin.

Band 1 of each toad resolved as a diffuse area on the starch-gel. Separation and staining characteristics of certain individual patterns indicated that this band in both *B. valliceps* and *B. fowleri* was a mixture of at least two proteins. For example,

the density of the stain was unevenly distributed throughout the band in most patterns of *B. valliceps* (Figs. 3 and 4). Its rate of migration was slightly faster in *B. fowleri* than in *B. valliceps*. Band 1 of the hybrid overlapped band 1 of both parental species.

Very little protein migrated in bands 2–5. These bands were frequently poorly resolved (Fig. 3). Band 2 was generally the most concentrated protein present in this region of the gel. It was constantly present in *B. fowleri* and absent in *B.*

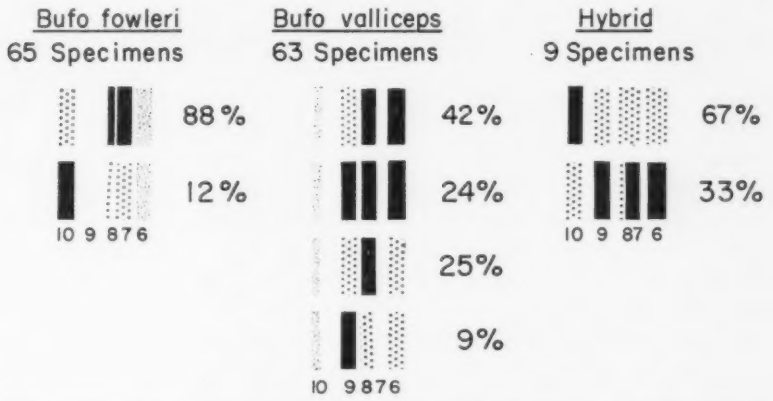

FIG. 5. Intraspecific variations in bands 6–10 of starch-gel patterns of the two species of toads and their hybrid. Percentages to the right of patterns represent their frequency of occurrence in animals examined.

valliceps. Similarly, band 3 was consistently found in *B. valliceps* and absent in *B. fowleri*. The area of these two bands was pale and diffuse in hybrid patterns. Band 4 was generally pale though consistently present in *B. fowleri*, absent in *B. valliceps*, and irregularly detectable in the hybrid. Band 5 was diffuse in *B. valliceps*, *B. fowleri* and the hybrid.

Patterns of protein bands 6–12 were most useful in distinguishing the toads from each other. The bands in this area of the gel were clearly resolved in most cases and were relatively highly concentrated when compared to bands in other areas of the patterns. The greatest percentage of plasma protein migrated in this area of the gel. The most common patterns of these bands in the two species and the hybrid are shown in Fig. 4. Band 6 was broad in all forms, of high concentration in *B. valliceps*, moderate concentration in the hybrid, and weak in *B. fowleri*. On some gels this band of the hybrid migrated slightly faster than band 6 of *B. valliceps* or *B. fowleri* (Fig. 3). Band 7 was generally of high concentration in *B. fowleri*, of moderate concentration in the hybrid and absent in *B. valliceps*. Band 8 was highly concentrated in nearly all specimens of *B. valliceps*. Although it was probably constantly present in *B. fowleri* and the hybrid, band 8 was often incompletely resolved from band 7 in these forms. Band 9 was of moderate concentration in *B. valliceps* and the hybrid but was absent in *B. fowleri*. Band 10 was broad and of

moderate concentration in *B. fowleri* and high concentration in the hybrid but was represented by only a very weak, narrow band in *B. valliceps*. Band 11 was somewhat diffuse but of higher concentration in *B. valliceps* and the hybrid than in *B. fowleri*. Band 12 was a narrow band of high concentration in each of the three forms.

Variations from these common patterns in the concentration of the bands occurred in the region including bands 6–10. Fig. 5 illustrates the variations and their frequency of occurrence. Four patterns were noted in *B. valliceps* but only two in each of the other forms. Repeated examinations of plasma from the same specimens of *B. fowleri* and *B. valliceps* at different times during a 6 month period indicated that neither bands 6–10 nor the remainder of the patterns was altered by the effects of captivity. No correlation was noted between the pattern of bands and the sex of the toad.

The position of the iron transport protein(s) was determined by autoradiography after electrophoresis (Fig. 6). The transferrin of *B. fowleri* migrated in band 7 and that of *B. valliceps* in band 11 (Fig. 4). No intraspecific variation in transferrins was noted in the animals examined (fifteen *B. fowleri*, nine *B. valliceps* and three hybrids). Plasma of the hybrids contained two transferrins, one which migrated at almost exactly the rate of *B. fowleri* transferrin and another which migrated at almost exactly the same rate as *B. valliceps* transferrin.

DISCUSSION

Hemoglobins

Paper electrophoresis revealed that the parent species of toads possessed hemoglobins with markedly different migration rates. No individual variation in hemoglobin mobility was detected in either species. The hemolysate of the hybrid usually separated on paper as a broad band, intermediate in mobility between the hemoglobins of the parent species. This broad band often appeared to consist of two fractions but these were never clearly separated. The hemoglobins of the parent species migrated with distinctly different mobilities in moving-boundary electrophoresis; hemoglobins of the hybrid migrated as a single, slightly skewed peak of intermediate mobility. An artificial mixture of hemoglobins from the parent species behaved similarly. On starch-gel electrophoresis the hybrid was shown to possess two hemoglobins, each apparently corresponding to that of one of the parent species, but each with slightly altered mobility (Fig. 1). Since neither the artificial nor natural (hybrid) mixtures of hemoglobins separated distinctly on paper or in free-solution electrophoresis, it would appear that a physical interaction between the two hemoglobins occurred as was postulated for mixtures of turtle hemoglobins (Dessauer *et al.*, 1957; Ramirez & Dessauer, 1957).

Plasma proteins

Paper electrophoresis suggested that the fast moving fractions of plasma proteins of *B. fowleri* migrated faster than those of *B. valliceps* (Fig. 2). The high resolution buffer gave a much sharper separation of protein bands than the barbital

buffer. Even with the high resolution buffer, however, the differences between the patterns of the two species were not always distinct and the fast migrating fractions of the hybrid were not clearly separated (Fig. 2). Possibly some of our difficulty in analysing the paper patterns was a result of individual variability. However, the lack of resolution in the patterns of the hybrid could be explained as follows: the fast migrating fractions of the two species did not quite coincide in mobilities, *B. fowleri* fractions being generally somewhat faster than those of *B. valliceps*. The interpretation that the hybrid inherited all the fractions from both species, could account for the patterns of the hybrid observed on paper; i.e. a pattern of interdigitating or overlapping fractions from both parent species in such a way that the peaks of one species filled up the troughs of the other. Interaction between proteins may also account for the poor resolution of the hybrid pattern by paper electrophoresis.

Starch-gel patterns were more reproducible; and, not only were several additional fractions differentiated in these patterns, but also more individual variability was revealed within each species and the hybrid than could be ascertained by the paper method. These variations appeared as differences in the intensity of certain bands. No individual variations in mobilities were found in either species (Fig. 5).

B. fowleri and *B. valliceps* differed markedly in starch-gel patterns of their plasma proteins. Outstanding were the different positions of the transferrins (Fig. 6). Transferrins within each parent species proved to be of constant mobility. This is surprising since transferrins undergo marked individual variations in many species within single populations including another species of toad, *Bufo terrestris* (Dessauer, Fox & Hartwig, unpublished). Also, there were several examples of protein fractions present in one species and absent in the other (Figs. 3 and 4). The two species also had a few fractions in common. Although the broad lead fractions of the two overlapped almost completely, the regions of greatest concentration did not correspond (Figs. 3 and 4).

The hybrids had apparently inherited components of all electrophoretic fractions from both parents (Figs. 3 and 4). Two transferrins, one from each parent species, were present in the hybrid (Fig. 6). Band 9 of *B. valliceps* was absent in *B. fowleri* but was present in the hybrid; band 7 occurred in *B. fowleri* and the hybrid but was absent in *B. valliceps*. The individual variation of patterns among these F_1 hybrids probably depended upon the particular pattern of their respective parents. Since the hybrids were captured wild, the individual patterns of their actual parents were not known.

General review

Chemical inheritance by hybrids has been studied by several methods. These include serological identification of red cell antigens (Landsteiner & van der Scheer, 1924; Irwin, 1932; Irwin & Cole, 1936; McGibbon, 1944; Bryan & Miller, 1953) and serum antigens (Walsh, 1924; Sokolovskaia, 1936; Sasaki, 1937; Cumley & Irwin, 1940; Boyden, 1942), immuno-electrophoresis of seed proteins (Hall, 1959),

moving-boundary electrophoresis of serum proteins (Brandt *et al.*, 1952), paper electrophoresis of egg-white proteins (Sibley, 1960) and chromatography of flower petal extractives (Turner & Alston, 1959).

These studies showed that all or most of the chemical components of both parents are inherited by their hybrid. In all cases studied the two parent species contained certain substances in common and, with one exception (chicken × pheasant, Brandt *et al.*, 1952), these substances were always present in the hybrid. Each parent species contained a number of components absent in the other species. Such "species-specific" components from both parents were present in hybrids of crosses between the Guinea hen and white leghorn cock (Sasaki, 1937), between two species of pigeons (Bryan & Miller, 1953), and between two species of legume (Turner & Alston, 1959). In other instances the hybrid lacked a number of components specific to one parent and thus resembled its other parent more closely. This was true for hybrids of crosses between chicken and pheasant (Brandt *et al.*, 1952), two species of doves (Irwin & Cole, 1936), horse and donkey (Landsteiner & van der Scheer, 1924; Boyden, 1942) and wheat and rye (Hall, 1959).

New hybrid components

A new "hybrid substance," not present in either parent species, was detected in certain hybrid studies (Irwin & Cole, 1936; Sokolovskaia, 1936; Brandt *et al.*, 1952; Bryan & Miller, 1953; Turner & Alston, 1959). No hybrid component was found in the investigations of Sasaki (1937), Cumley & Irwin (1940) and Hall (1959). In general, patterns of our hybrid toads gave little indication of the presence of a "hybrid substance." However, in the plasma pattern of one hybrid (Fig. 3) fraction 6 migrated faster than fraction 6 of either *B. valliceps* or *B. fowleri*. This variation was consistently reproduced on three separate electrophoretic analyses of three different plasma samples from this individual.

Most examples of "hybrid substance" reported were non-protein products synthesized in biochemical reactions catalysed by protein enzymes, i.e. cellular antigens and flower extractives (Allison, 1959). To our knowledge the only investigations in which proteinaceous "hybrid substances" were detected were the electrophoretic studies of Brandt *et al.* (1952) and the serological studies of Sokolovskaia (1936). The authors have not seen Sokolovskaia's publication. Brandt's work showed that protein–protein interaction caused alterations of mobilities of plasma proteins of hybrid birds and probably accounted for apparent "new fractions."

Although newly formed "hybrid proteins" appear to occur only rarely, they might be expected more commonly in view of one current hypothesis of gene action (Ingram, 1960). Briefly this hypothesis suggests that the product of the action of a dominant gene is a specific peptide. Once synthesized this peptide combines with other peptides produced by the same gene or its homologous allele. The resulting product is a specific protein. Presumably wild species contain many homozygous, dominant genes. A hybrid would inherit most if not all of the dominant genes of the two parent species. The action of these genes would yield peptides common

to both parents as well as specific to each parent. In such a hybrid mixture polymerization of random combinations of peptides should form proteins with properties different from those of either parent as well as proteins specific to each parent. Our data, however, indicate that proteins of the hybrid are electrophoretically identical to those of their parents. On the other hand, the commonly found "hybrid substances" are non-proteinaceous and are several steps removed from direct genic control. The probability of such "hybrid substances" being formed seems great.

High plasma protein levels and hybrid vigor

Volpe (1960) found that tadpoles of the hybrid between crosses of male *Bufo valliceps* and female *Bufo fowleri* grow faster and metamorphose earlier and at a larger size than uncrossed tadpoles of either species. Wild captured adult hybrids survived better in captivity than adults of the parent species. The correlation of these evidences of hybrid vigor with the high total plasma protein levels of these hybrids is very suggestive. Such increased growth and developmental rates and high plasma protein would appear to indicate that these hybrids were better equipped through their gene complement to perform protein synthesis than either parent species. This could be explained on the assumption that the hybrid inherits most of the dominant genes from both parent species.

SUMMARY

1. Hemoglobins and plasma proteins of two species of toads and their natural (F_1) hybrid were compared electrophoretically.

2. Hemoglobins of the two species migrated at different rates. The hybrid inherited two hemoglobins, each of which only approximated the mobility of hemoglobin of one of the parent species. Artificial mixtures of the two parent hemoglobins showed similar modifications of mobilities suggesting that a physical interaction between proteins was involved.

3. Total protein concentration of the plasma was significantly higher in the hybrid than in its parents.

4. On paper electrophoresis the fast moving fractions of the plasma proteins of the two species of toads migrated at slightly different rates. The hybrid plasma pattern appeared to be a composite of those of the parent species.

5. Twice as many plasma protein fractions were resolved by starch-gel as by paper electrophoresis. The two species of toads possessed several fractions in common; frequently these were of different concentrations. Plasma of each species also contained several fractions not found in the other species. Considerable individual variation in the quantity of certain fractions occurred within each species and the hybrid.

6. Each species possessed a single transferrin. The transferrins of the two species migrated in widely separated protein fractions. The hybrid possessed two transferrins, each with a mobility almost identical to that of one of its parents.

7. The hybrids appeared to inherit protein of all the plasma fractions of both species. With one possible exception, no protein was found in hybrid blood that was not present in one or both of the parent species.

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THE EFFECT OF TEMPERATURE CHANGES ON THE RESTING POTENTIAL OF CRAB, INSECT AND FROG MUSCLE*

G. A. KERKUT and R. M. A. P. RIDGE

Department of Physiology and Biochemistry, The University of Southampton

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Abstract—1. The Q_{10} was determined for the resting potential of muscle fibres from the crab *Carcinus maenas*, the cockroach *Periplaneta americana* and the frog *Rana temporaria*.

2. The Q_{10} values were: crab, $1{\cdot}064 \pm 0{\cdot}004$; cockroach, $1{\cdot}296 \pm 0{\cdot}046$; frog, $1{\cdot}060 \pm 0{\cdot}004$.

3. The crab and frog Q_{10} were very much alike and significantly different from that of the insect.

4. Individual muscle fibres in the crab had significantly different Q_{10}'s. The same was true for individual muscle fibres in the frog.

5. The resting potential took longer to adjust to a temperature decrease than it did a temperature increase.

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INTRODUCTION

THE FACT that there is a steady potential difference between the inside and outside of nerve and muscle cells is well established, but the precise manner in which this potential is brought about and maintained is not yet fully understood. There are two main views concerning the relationship between metabolic systems and the resting potential. One suggests that metabolic systems are clearly important but their action is secondarily related to the resting potential by their maintenance of an asymmetrical ionic distribution across the membrane (Hodgkin, 1951, 1957; Conway, 1957). The other view suggests that the metabolic activity is directly concerned with the resting potential (Lorenté de No, 1947; Ling & Gerard, 1949; Ling, 1952; Grundfest, 1955). An investigation into the manner in which the resting potential responded to sudden temperature changes might indicate the relationship between the resting potential and a simple diffusion potential.

MATERIALS AND METHODS

The experiments were carried out on whole muscles *in situ* on the isolated limb, dissection being kept to the minimum. In *Carcinus* the leg was removed from the body and the exoskeleton removed from the meropodite to reveal the carpopodite extensor and flexor muscles. In *Periplaneta*, a leg was removed and a piece of the exoskeleton removed from the femur thus exposing the fibres of

* This work was done with the aid of a grant from the Department of Scientific and Industrial Research and the Royal Society.

the flexor and extensor tibialis. In *Rana*, the forelimb was removed from the body and a muscle on the upper forelimb used. The preparation was secured to a glass slide and placed in a small muscle bath. The bath was constructed so that saline of a specific temperature could flow over the muscle without disturbing the muscle-electrode system, yet at the same time allowing a fairly rapid change of temperature. 3 M KCl-filled glass microcapillary electrodes of 5–30 MΩ resistance were tested for temperature sensitivity in the saline used, and only those showing a voltage–temperature change of less than 1 mV for a 10° change were selected. The electrode was connected by silver–silver chloride to an electrometer valve input (Belton, 1958) and thence to a Southern Instruments d.c. amplifier which drove a pen on an Ediswan pen recorder. The output was monitored on an oscilloscope. The muscle bath was connected through an agar–saline bridge to a saline container which led via a silver–silver chloride connexion to a calibrator and thence to earth. This was found necessary as otherwise the junction potential of the system proved to be sensitive to temperature changes. The temperature of the bath was recorded by means of a Stantel thermistor inserted in close proximity to the muscle. The thermistor formed the fourth arm of an unbalanced Wheatstone Bridge and the output of the bridge was connected to a d.c. power amplifier and thence to the second pen of the recorder. The temperature range of the system was initially adjusted for the range 15–25°C and the response of the thermistor was practically linear, though it was check calibrated after each experimental run.

The composition of the Ringer solutions used is given in Table 1.

TABLE 1—COMPOSITION OF THE RINGER SOLUTIONS (g/l)

	Crab (Fatt & Katz, 1953)	Insect (Boistel, 1960)	Frog
NaCl	30·0	8·9	6·0
KCl	0·96	0·19	0·076
$NaHCO_3$	0·22	0·096	0·1
$CaCl_2$	1·3	0·2	0·1
$MgCl_2$	2·25	—	—
Glucose	—	4·0	—

The procedure used was as follows. A muscle fibre was speared with a microelectrode and its resting potential measured. Only stable potentials were used. The temperature of the bath was then altered from 15 to 25° and the potential change and temperature change recorded on the pen recorder. The resting potential was allowed to reach a steady potential after each temperature change. This procedure was repeated many times for each muscle fibre. The resting potential was calibrated and the temperature response of the thermistor checked after each series of experiments. The experimental resting potential and temperature

values could then be read from the paper record. It usually happened that the bath temperature was not exactly 15° but say 14·9° or 15·1° and a similar variation occurred at the upper temperature. A graph of temperature–resting potential was constructed for each series of changes carried out on one experimental muscle fibre, so that a straight line linked each pair of temperature change. From this series of lines it was possible to draw an average line from which one could read the value of the resting potential at exactly 15° and 25° and in this way obtain a value of Q_{10}. All values are given together with the standard error.

RESULTS

1. *The resting potential of muscle fibres*

(a) *Carcinus maenas.* The resting potential of the crab muscle fibres immersed in crab Ringer was $63{\cdot}4 \pm 1{\cdot}4$ mV. This value is somewhat higher than that obtained by Shaw (1955) who immersed the muscle fibres in crab blood and obtained a value of 58 mV. Fatt & Katz (1953) working on the crabs *Portunus depurator* and *Carcinus maenas* found that the resting potential of muscles immersed in crab Ringer ranged from 56 to 90 mV with a mean of $70 \pm 0{\cdot}5$ mV.

(b) *Periplaneta americana.* The resting potential of cockroach muscle fibres immersed in cockroach Ringer was $56{\cdot}5 \pm 1{\cdot}7$ mV. Wilson (1954) using the flexor tibialis of the cockroach found that this muscle immersed in Roeder's saline showed resting potentials ranging from 30 to 70 mV with a mean of 45 mV.

(c) *Rana temporaria.* The resting potential of frog muscle fibres immersed in frog Ringer solution was $98{\cdot}3 \pm 3{\cdot}8$ mV. Ling & Gerard (1949) found that the resting potential of the sartorius muscle was $78{\cdot}4 \pm 5{\cdot}3$ mV but that over runs of 200 experiments using an optimal external potassium concentration the value was $97{\cdot}6 \pm 5{\cdot}7$ mV.

2. *Temperature changes on the resting potential*

The electrodes were inserted into a superficial muscle fibre of the animal in question and the absolute value of the resting potential determined. This potential was then backed off and the gain of the amplifier increased so that full scale deflexion of the pens could be brought about by a change of approximately 10 mV. For most of the readings one could detect a change of 0·25–0·5 mV in the resting potential.

The preparation was allowed to settle down for a few minutes to make sure that there was no large drift in the system, and then Ringer solution of a specified temperature was passed through the bath. The bath took a maximum of 10 sec to reach the new temperature though in most cases equilibrium was reached more rapidly. This temperature was maintained for a time and then a fresh supply of Ringer at another temperature was passed through the bath. In this way the muscle was treated to an increase and then a decrease of temperature. During the course of any one experiment, the temperature was changed several times and the variation in resting potential noted. At the end of the experiment the absolute

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resting potential was once more determined to allow one to ascertain whether the resting potential had fallen and if the zero on the recording system had drifted. All unsatisfactory cases were rejected.

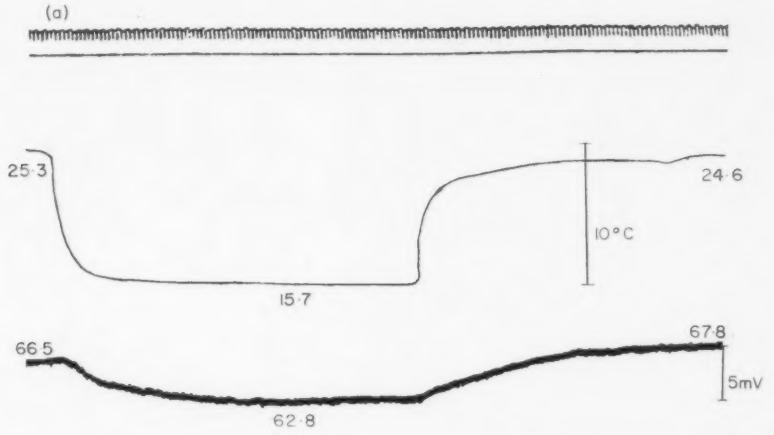
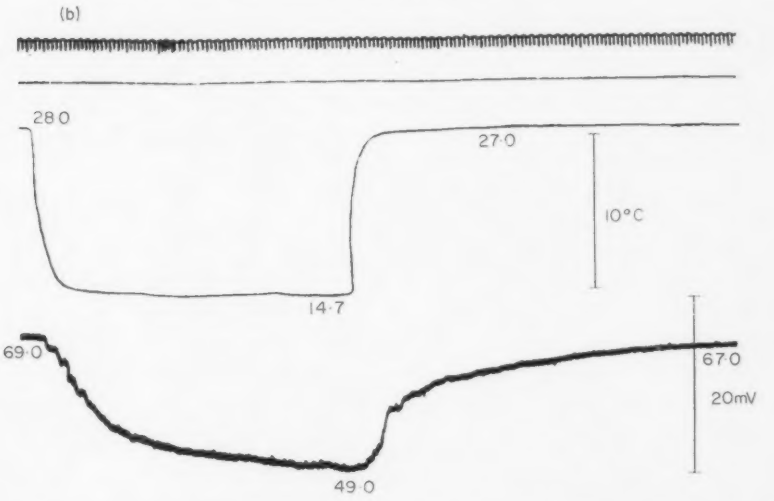


FIG. 1. The effect of temperature changes on the muscle resting potential. (a) Crab, (b) Cockroach, (c) Frog. Each record shows in order: time base with intervals of one second marked; base line; temperature record; resting potential. The numbers indicate the temperature and resting potential values.



It will be seen from Fig. 1 that the resting potential followed the temperature variation quite well. The resting potential increased for an increase in temperature and decreased for a decrease in temperature. The change was of the order of 5 mV per change of 10°C and the resting potential took from 10 to 40 sec to reach

equilibrium. In the frog and the crab the resting potential took one third longer to reach an equilibrium value following a decrease in temperature than following an increase in temperature. This difference was even more marked in the case of insect muscle, where it took almost twice as long.

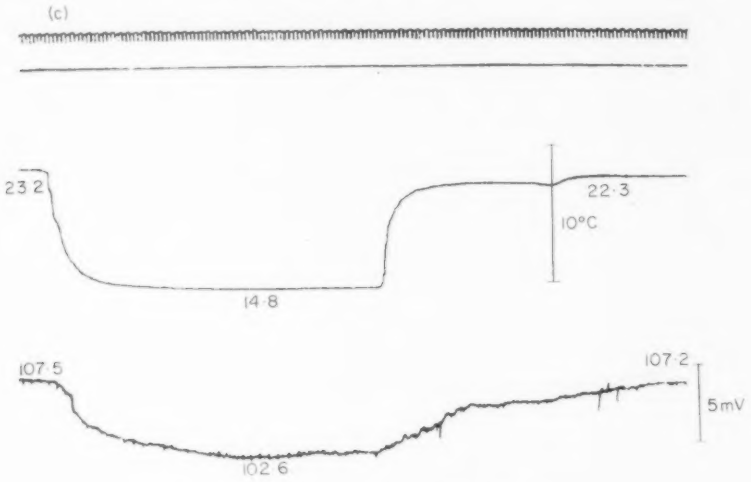


FIG. 1 (contd.). For caption see p. 67.

The Q_{10} estimations were derived from approximately 100 experiments on each type of muscle. The value for crab muscle was $1{\cdot}064 \pm 0{\cdot}004$. It is of interest to note that there was a significant individual difference of Q_{10} between muscle fibres of the same crab. Thus one individual crab showed one muscle fibre with a Q_{10} of $1{\cdot}054 \pm 0{\cdot}002$ whilst another fibre in the same muscle gave a Q_{10} of $1{\cdot}081 \pm 0{\cdot}003$ ($P < 0{\cdot}001$).

The Q_{10} value for frog muscle was $1{\cdot}060 \pm 0{\cdot}004$. This value is practically identical with that obtained for crab muscle, the difference between the two not being significant. As in the case of crab muscle, individual frog muscle fibres showed a significant difference in their Q_{10}. Thus one fibre had a Q_{10} of $1{\cdot}080 \pm 0{\cdot}005$ whilst another fibre gave a Q_{10} of $1{\cdot}043 \pm 0{\cdot}001$ ($P < 0{\cdot}001$).

Insect muscle gave a Q_{10} of $1{\cdot}296 \pm 0{\cdot}046$. This value is much higher than that obtained for crab or frog muscle and is significantly different from these. Thus the probability of obtaining the insect Q_{10} during either the frog or crab experiments was $< 0{\cdot}001$.

DISCUSSION

The values of the resting potentials described in this paper agree fairly well with those obtained by previous authors. It is interesting to note that our results for *Carcinus* agree better with those obtained by Fatt & Katz (1953) who also used crab Ringer solution, than those obtained by Shaw (1955) who used crab blood. This might be taken to indicate that crab blood had a higher potassium concentration

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than did crab Ringer, but Shaw's analysis of crab's blood gave 12·1 m equiv K/l whilst the Ringer solution had 12·9 m equiv K/l. It is quite likely that some other factors in the blood may be of importance in determining the precise value of the resting potential.

If the resting potential was mainly the result of an asymetrical distribution of potassium and chloride ions as expressed by the Nernst equation, then the resting potential, under physiological conditions, should follow the temperature so that the Q_{10} over the range 15–25°C would be equal to 1·035. The values that we obtained in these experiments were $1{\cdot}064 \pm 0{\cdot}004$ (crab), $1{\cdot}296 \pm 0{\cdot}046$ (insect) and $1{\cdot}060 \pm 0{\cdot}004$ (frog). From the level of the standard errors it can be seen that all three of these are significantly different from the theoretical Q_{10} value of 1·035. In all three cases $P < 0{\cdot}001$.

It will be noted that in all three cases the observed Q_{10} value was higher than the theoretical value, the difference being greatest in the case of insect muscle. Significant differences were also noted between muscle fibres in the same muscle although the resting potential of these muscle fibres was almost identical. The Q_{10} values obtained here differ from those obtained by Macfarlane & Meares (1958) for the isolated sartorius muscles from the amphibians *Hyla coerulea* and *Bufo marinus* where the Q_{10} was 1·03 for the ranges 0–20°C and 30–40°C and 1·05 for the range 20–30°C. It is possible that our higher values could be due to some extent to the fact that we were working over the range 15–25°C, a range still within the natural limits of the animals. Macfarlane & Meares allowed their tissues to equilibrate from 10 to 30 min at the new temperature. In our experiments we were more concerned with a rapid change in resting potential with time and the maximum time taken for the resting potential to reach its equilibrium potential was 40 sec. Ling & Woodbury (1949) reported a Q_{10} of 1·1 for frog muscle in normal Ringer solution between 4 and 30°C. However, with a Ringer solution containing a higher concentration of potassium (5 mM K^+) and M/150 phosphate, they obtained a Q_{10} of 1·033. They suggested that this Ringer solution stopped potassium leakage and hence allowed the system to obey the Nernst equation.

The resting potential of muscle fibres may differ from that of nerve in its variation with temperature. Hodgkin & Katz (1949) found that the resting potential of squid axons was almost independent of temperature between 2 and 20°C. On the other hand Burkhardt (1959), working on the crayfish stretch receptor, showed that the resting potential decreased with an increase in temperature.

We have recently repeated the experiments of Lund & Moorman (1931) on the determination of the Q_{10} for the frog skin potential. They obtained a Q_{10} of 1·97 whilst we obtained a similarly high value of 1·75. (We differed from Lund & Moorman in that they observed a considerable time lag, up to 40 min, between the change of temperature and the full adjustment of the skin potential. We did not observe any such lag in the reaching of a stable potential.) These Q_{10} values are much higher than those calculated from the Nernst equation and the frog skin potential is now regarded as a potential maintained by active metabolism rather than as a simple diffusion potential. The higher Q_{10} values reflect this.

In this respect it is of interest to note that the Q_{10} for crab, insect, and frog muscle, obtained in the present experiments, is significantly higher than that derived from the Nernst equation.

It is difficult to know the precise significance of Q_{10} measurements; thus the concept that physical systems will have a Q_{10} of 1, whilst chemical systems will have a higher Q_{10} is clearly an oversimplification. Nevertheless, Q_{10} measurements have some value especially when measured on apparently similar systems such as muscle fibres in different groups of animals or nerve and muscle fibres.

The results reported in this paper do offer suggestive evidence that the resting potential of muscle fibres is a complex phenomenon linked with metabolic activity, rather than a simple physical diffusion potential.

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BOOK REVIEW

L. LISON: **Histochimie et Cytochimie Animales.** 3rd ed., Vol. 1. Gauthier Villars, Paris, 1960. 397 pp., 45 N.F., $9·50.

IT IS NOW almost 25 years since the publication of the first edition of Lison's *Histochimie Animale*, an event which it may fairly be claimed, first placed histochemistry on a sound basis. Since the time of Raspail little histochemistry had been attempted—such as there was involved a more biochemical approach and consequent destruction of the tissue. Moreover, with the advent of the aniline dyes in the latter part of the nineteenth century, attention was diverted to the staining of tissues with the object of determining their structure and the chemical nature of these tissues was completely neglected. This outlook was entirely changed by the work of Lison, who introduced a critical approach towards histochemical methods and by determining their validity, limits and uncertainties, opened the way to an understanding of how the structure of tissues could be linked to a knowledge of some aspects of their chemical nature.

Since the first edition of this book, histochemistry has progressed greatly and it is indeed a pleasure to welcome the third edition of such a classic text. This edition represents a completely new book, since the results of so much research have been incorporated into the subject in recent years that a revision of the existing text obviously proved impracticable. This new edition of the *Histochimie* is split up into two volumes (of which only the first has yet appeared); this first volume deals with general principles and the validity of methods, with physical and optical methods, with the reactions of individual radicals and with the special reactions of the proteins and nucleic acids.

The section on general principles is extremely valuable, as so many workers attempt to utilize the techniques of histochemistry with little knowledge of the many problems and pitfalls involved in this approach. In particular the author emphasizes the dual nature of the histochemical problem—the discovery of the identity of a substance and secondly its localization in the cell or tissue. Such a problem may be attacked in two quite separate ways. There is the standard biochemical approach, with its great refinement of technique for discovering the identity of a substance but with limitations to its ability to give information as to the location of that substance in the cell. The alternative is the use of *in situ* histochemical methods which suffer from poor characterization of compounds but do give more useful information as to their location. In this book, although the author does not attempt to deal with the *extra situm* or more biochemical approach, he does emphasize the necessity for the use of both types of approach in the attempted solution of any problem. Any such closer collaboration between histochemists and biochemists will surely be very fruitful; the present reviewer welcomes the way in which this is made clear by the author, many histochemists still believing that they have nothing to gain from such collaboration.

One completely new section of the book is devoted to the reactions of radicals or reactive groups. Much of current histochemistry is in fact the detection of the presence of such groups rather than that of actual compounds. It is, one feels, a very valuable step forward to see this principle recognized in a leading text book. A further improvement noted in this edition is the summarizing in tabular form of the reactions of each group of substances at the beginning of the chapter in which they are discussed. This is perhaps a minor point, but one which contributes to the value of the book as a whole.

As we have come to expect from this author, the book has many references and is written in a very clear manner. The fact that the language is not our own should not deter intending

users! The value of the contained information is such as to justify any struggles with the French. All details of actual techniques are now to be included in a separate section in Volume 2, so that at present the book cannot be used as a laboratory manual; other books are, however, available to fill this purpose and the majority of histochemists will find that Lison is more often consulted in the study than in the laboratory. One hopes that the publishers will produce the second volume of this work with the minimum delay. In short, this is a book which can be unreservedly recommended.

S. BRADBURY

ERRATUM

C. A. G. WIERSMA and R. L. C. PILGRIM: Thoracic stretch receptors in crayfish and rock-lobster. *Comp. Biochem. Physiol.* **2**, 51–64.

In the Abstract item (3) should read:

(3) It is shown by electrical recording and by staining that each of the first five segmental thoracic nerves is connected to a single receptor cell (N-cell) which is not a specialized receptor muscle; the sixth nerve in *Procambarus* innervates a single slow stretch receptor, and in *Panulirus* a fast one in addition.

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Comp. Biochem. Physiol., 1961, *Vol.* 3, *pp.* 73 *to* 91. *Pergamon Press Ltd., London. Printed in Great Britain*

THE MECHANICAL AND COLLOIDAL PROPERTIES OF *AMOEBA* PROTOPLASM AND THEIR RELATIONS TO THE MECHANISM OF AMOEBOID MOVEMENT

K. YAGI

Zoological Institute, Faculty of Science, University of Tokyo

(*Received* 18 *November* 1960)

Abstract—1. A nickel particle introduced into the endoplasm of *Amoeba* (*proteus* type) was moved by magnetic force and the relation between the local viscosity and the flow rate of endoplasmic sol was studied. In general the higher the streaming rate was, the lower was the viscosity. The viscosity ranged from about 0·1 poise to about 3 poise. Slowly flowing endoplasm sometimes exhibited low viscosity.

2. An anteroposterior gradient of viscoelasticity of ectoplasmic gel was found in moving amoebae.

3. The ectoplasmic gel has also the property of retarded elasticity.

4. By measuring the changes in the distance between two granules the three-dimensional contraction of the ectoplasmic gel in moving amoebae was ascertained. The contraction rate was the highest in the tail region where the ectoplasm solates.

5. The fact was revealed that solation of *Amoeba* protoplasm is always accompanied by an increase in volume of a mean value of 57 per cent. Volume decrease in gelation is not necessarily correlated with water discharge through the pulsating contractile vacuole. It is probable that water may be eliminated directly through the cell surface.

6. The theory of amoeboid movement based on ectoplasmic contraction and the theory of diffusion drag force are discussed. The significance of the solating reaction in amoeboid movement is emphasized.

INTRODUCTION

MANY investigations have been made into the problem of the mechanism of amoeboid movement. Hyman (1917) designated the possible significance of sol–gel transformation of protoplasmic colloid in amoeboid movement. More recent work (Pantin, 1923; Mast, 1931; Goldacre & Lorch, 1950) has favoured some form of contractility as the motive force of endoplasmic streaming. Stewart & Stewart (1959) have advanced a diffusion drag force hypothesis which is based upon detailed observations of protoplasmic movement in slime mould plasmodia. However, none of these theories appears to have been based upon quantitative observations. In the present study the author has attempted to provide quantitative measurements of the local mechanical properties of normal *Amoeba* protoplasm by applying magnetic force to nickel particles ingested by the organism. The volume changes which occur with changes in the colloidal state of protoplasm also were studied. The significance of these mechanical and colloidal properties for amoeboid movement is discussed.

MATERIAL AND METHOD

Amoebae of the *proteus* type which were fed on *Tetrahymena geleii* in inorganic culture solution containing 0·08 mM, KCl; 0·3 mM, $CaHPO_4$; and 0·02 mM, $MgSO_4$ (pH 6·7, temp. 20–25°C) were used as study material. Minute ellipsoidal nickel particles were ground together with *Tetrahymena* in an agate mortar. The nickel particles were coated with cytoplasmic substance of *Tetrahymena*, so as to make them easily ingested by amoeba. The particles were fed to a culture of amoeba, which had been previously starved overnight. A few hours after such feeding, some of the nickel particles ingested by the amoebae were found in the protoplasm. The measurements were made exclusively on those particles, which were observed lying naked in the protoplasm or contained within a food vacuole so small as to leave almost no space below the vacuolar membrane. Use of particles within larger vacuole was avoided to eliminate the possible effect of movement within the vacuole which might affect the results significantly. Such states of the particles were confirmed by microscopic observation with high magnification as well as by observing that the particle was not moved within the vacuolar space by weak magnetic force, when it was in the ectoplasmic gel.

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The apparatus producing the magnetic force and the other details of measurements are described in the following parts of the paper.

RESULTS

(1) *Mechanical properties of endoplasmic sol*

Mechanical properties of living endoplasm could be studied by analysing the movement of the nickel particle induced by the magnetic force. A horizontal U-shaped electromagnet was used. In order to obtain a steep gradient of density of magnetic flux, one of the faces of the pole pieces was made convex, and the other concave, both being kept homocentric. The axis of the magnet was fixed at a right angle to the optical axis of the vertical microscope. The amoeba containing a single nickel particle (2–9 μ in the longest diameter) within the endoplasm was introduced into a glass capillary to oblige the organism to become monopodal, and move parallel to the capillary wall. The capillary was placed horizontally and at right angles to the axes of both magnet and microscope. Thus, the nickel particle moved horizontally within the endoplasmic sol and it was pulled magnetically in the horizontal plane at a right angle to the direction of endoplasmic streaming.

Movement of the particle was recorded on 35 mm photographic film at three frames per second, and a typical result is shown in Fig. 1. It is seen in this figure that the particle moved with almost uniform velocity during the application of the magnetic force and that no elastic return occurred just after removal of the force. Therefore, the endoplasmic sol of the living amoeba seems to have almost no elastic property, but to be of a purely viscous nature. The viscous property of the endoplasm might be measured from the velocity of movement of the particle due to magnetic force. However, the rate of horizontal movement of the nickel particle in such experiments seems to be influenced by two factors; one is the viscosity of the endoplasmic sol, and the other is the friction between the heavy nickel particle

on the bottom of the endoplasmic sol and the surface of the ectoplasmic gel. In the following Section measurements of the viscosity of the endoplasmic sol were made by moving the nickel particle vertically, in order to avoid the difficulty in interpretation introduced by the second factor mentioned above.

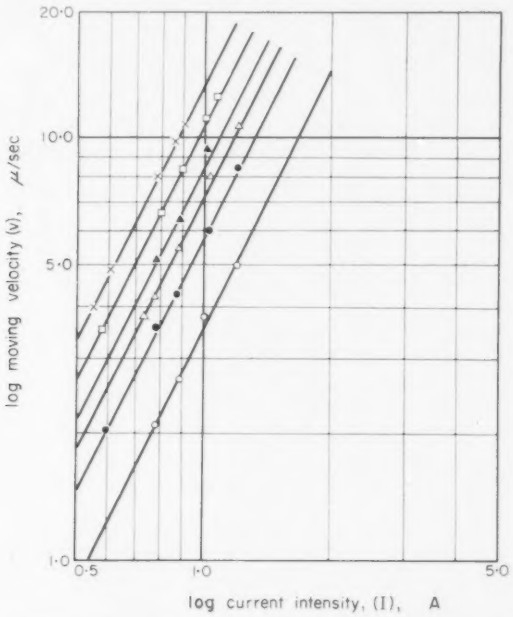

FIG. 2. Relation between intensity of current flowing through magnetic coil and moving velocity of nickel particle in a glycerol solution with a viscosity of 0·33 poise. Each of the different symbols in the figure represents the data for a different particle.

(2) *The viscosity and the streaming rate of endoplasmic sol*

An electromagnet of a rod type having two conical pole pieces was used. The axis of the magnet was fixed vertically to the optical axis of a horizontal microscope so that the both axes intersected with each other at a point 1·0 cm from the lower surface of the pole. The nickel particle which was being moved horizontally at the same time by endoplasmic streaming was pulled vertically upward by magnetic force. Consequently the particle was forced to move in a direction oblique to the cytoplasmic streaming direction during application of the magnetic force. The moving velocities of nickel particle due to the endoplasmic streaming and to the magnetic pulling were measured simultaneously by stop-watch timing the movement across a squared calibrated ocular micrometer. Thus, it was possible to calculate the absolute value of endoplasmic viscosity by use of the formulae whose derivation is described below.

The experimental formula and calibration indicating the relation between the intensity of electric current (I) flowing through the magnetic coil, the viscosity of the surrounding

fluid (η), the size factor (V/r) and the moving velocity (v) of nickel particle, was obtained by pulling the nickel particle in the standard solutions of glycerol of various concentrations. The size factor (V/r) of the particle represents the ratio of the product of the lengths of three main diameters to the radius of the cross-section perpendicular to the moving direction. This factor was found to be unchanged during the application of magnetic force to any particular particle. The instruments and procedure used in the determination were the

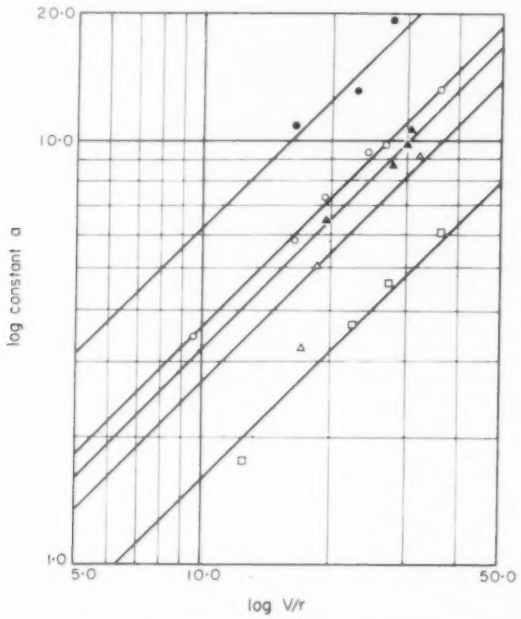

FIG. 3. Relation between the size factor (V/r) and the constant a. Lines represent the relations at different viscosities expressed by different symbols. Viscosity for ●: 0·11, ○: 0·17, ▲: 0·22, △: 0·33, □: 0·49 poise.

same as those used in the biological measurements. The absolute viscosity values of the calibrating standard solutions were measured by means of a hydrometer and Ostwald's viscosimeter. In each standard solution the moving velocities of several particles were measured by using the ocular micrometer and a stop-watch.

The results of one of the standarizations using solutions of known viscosity are shown in Fig. 2. It may be seen that the relation between the current intensity and moving velocity can be expressed by $v = aI^2$, where a is the constant depending on the size factor of each particle. As shown in Fig. 3 the constant a is directly proportional to the size factor (V/r), that is, $a = b(V/r)$, where b represents the constant specific to the viscosity value (η) of each standard solution. Concerning the relation between b and η of each standard solution, it was found that these vary inversely with respect to each other as shown in Fig. 4. Thus, introducing the constant obtained from this illustration, it is possible to express b as follows: $b = 7{\cdot}5 \times 10^{-2}/\eta$.

From the relations stated above the following experimental formula was obtained:

$$\eta = \frac{7{\cdot}5 \times 10^{-2}}{v} \cdot \frac{V}{r} \cdot I^2$$

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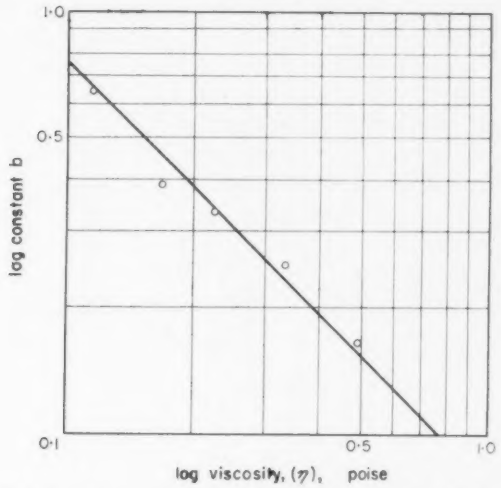

FIG. 4. Relation of constant b to the viscosity (η) of standard solutions.

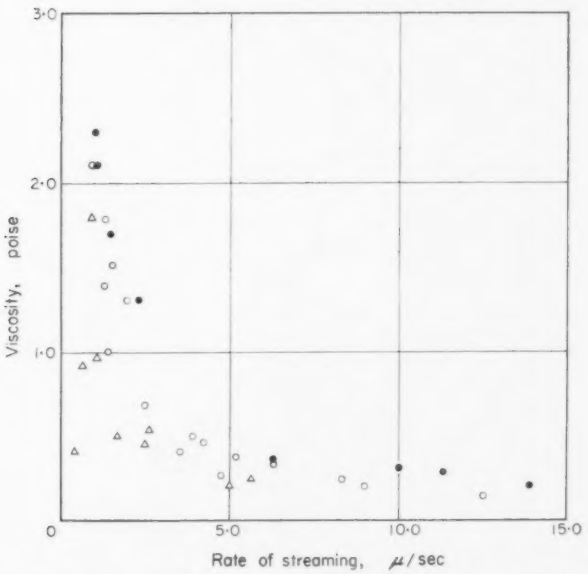

FIG. 5. Relation between the local viscosity and the streaming rate of the endoplasm. Different symbols indicate the different individuals.

This formula accompanied the mean percentage errors of about 13 per cent within the range of η obtained in the present experiment.

Values of I could be measured and were kept constant throughout the measurements on the same organism. V/r was also measured and was found to be constant during the magnetic pulling on each particle as mentioned previously. Therefore, η corresponding to each v-value was calculated from the above equation. The results of three examples are shown in Fig. 5. The viscosity values of the endoplasmic sol flowing at a lower rate were markedly higher than those streaming quickly. The changes in viscosity appear to be graded with the change in streaming rate. However, in some cases low values were obtained at very low streaming velocities (Fig. 5).

Cytoplasm is not a uniform medium, and many minute granules were observed suspended in amoeba protoplasm. Accordingly, the measured values of viscosity may be different from the viscosity values of granule-free cytoplasm. According to Einstein (1906) and Hatschek (1910), the relation between the apparent viscosity (η_c) of a fluid in which solid particles are suspended, and the viscosity (η_0) of the homogeneous fluid is expressed by $\eta_c = \eta_0 (1 + k \cdot f)$, in which k represents the constant which was estimated to be 4·5 by Hatschek and f is the ratio of the volume of dispersed particles to that of suspending fluid. In the present material the average of the volume of each small observed granule and of the dispersing density of the granules within the endoplasm were measured to be 4·6 μ^3 and $2{\cdot}1 \times 10^{-3}/\mu^3$ respectively. Hence, the value f in the present case should be 0·0097 or about 0·01. This fact seems to indicate that the actually obtained value of viscosity nearly represents the viscosity of the granule-free protoplasm.

Another possible source of error might be introduced by the collision of the nickel particle with protoplasmic granules during measurement. However, the expected number of collisions with observable granules calculated from the granule density mentioned above, and the space occupied by the nickel particle during the magnetic pulling, was from 0·01 to 0·46. Accordingly, the effect of such collisions may be negligible.

The experimental formula derived to fit the present case may be taken to be a modification of Stokes' law. The application of this law to this situation requires that Reynolds' number (R) should be less than 1 in c.g.s. unit (Deutsch, 1928). Reynolds' number may be represented by $R = 2rv\rho/\eta$, in which v represents the moving velocity of the nickel particle, r the radius of the particle measured vertically to the moving direction, η the endoplasmic viscosity and ρ the density of protoplasm. Adopting the value 1·05 of Heilbrunn (1926) as ρ, R for the present experiment was calculated to be between $7{\cdot}4 \times 10^{-7}$ and $4{\cdot}0 \times 10^{-6}$, or far less than 1.

If it is possible to regard the monopodal amoeba as an ectoplasmic tube within which the endoplasmic sol is passively forced to flow, then the pressure difference between both ends of the tube may be estimated from the Poiseuille's law. According to this law, the volume (V) of a liquid flowing out of a cylindrical tube per unit time is given by $V = \pi P r^4/81\eta$, in which P is the pressure difference between both ends of the tube, r and l are the radius and length of the tube respectively and η the viscosity. Since $V = \pi r^2 v$, in which v is the average velocity of flow through the tube, $v = Pr^2/81\eta$. Assuming that the endoplasm flows through a cylindrical tube of $1{\cdot}1 \times 10^{-3}$ cm in radius (r) and $3{\cdot}5 \times 10^{-2}$ cm in length (l), which are the average values actually measured on three specimens, the values of P may be calculated from the equation. The result of such calculations based upon a single

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organism is shown in Table 1, together with the values of η and v used for each calculation. In spite of rather wide variation in the value of η, the calculated value of P is more or less steady. This fact is important in connexion with the discussion to follow. Table 2 contains P-values obtained from different organisms. There is considerable individual variation in these values.

TABLE 1—SUCCESSIVE MEASUREMENTS OF PRESSURE DIFFERENCES BETWEEN THE OPPOSITE ENDS OF ONE AMOEBA DURING ENDOPLASMIC STREAMING

Streaming rate (μ/sec)	Endoplasmic viscosity (poise)	Pressure difference (dyn/cm^2)
1·1	2·1	54
1·1	2·3	59
1·5	1·7	60
2·3	1·3	70
6·3	0·35	52
10·0	0·31	73
11·3	0·28	74
13·9	0·21	68
	Mean	$64 \pm 8{\cdot}0$

TABLE 2—PRESSURE DIFFERENCE BETWEEN THE OPPOSITE ENDS OF SIX AMOEBAE ENGAGED IN ENDOPLASMIC STREAMING

Number of specimen	No. of measurements	Pressure difference (dyn/cm^2)
1	17	115 ± 25
2	8	$64 \pm 8{\cdot}0$
3	17	$45 \pm 7{\cdot}2$
4	3	$29 \pm 0{\cdot}9$
5	14	33 ± 12
6	6	74 ± 21

Heilbrunn's calculation (1952), a value of 8 dyn/cm^2 for pressure difference between both ends of streaming endoplasm in *Amoeba* is much lower than that found. On the other hand, Kamiya (1940) has shown that the force generated by a plasmodium of *Physarum polycephalum* reached a maximum value equal approximately to a 10 cm water column (or about $9{\cdot}8 \times 10^3$ dyn/cm^2). Allen & Roslansky (1959) have estimated the value of $5{\cdot}6 \times 10^3$ dyn/cm^3 as the pressure gradient in streaming endoplasm in *Chaos chaos*. This value is also higher than that obtained in the present study. From the present data in Table 2 the value of $3{\cdot}3 \times 10^3$ dyn/cm^3 can be calculated as the maximal pressure gradient observed by us in *Amoeba*.

(3) *Viscoelasticity of ectoplasmic gel*

In the following experiment the viscoelasticity of ectoplasmic gel was studied with reference to protoplasmic streaming. The electromagnet used was the same as that used in the first experiment.

The amoeba having a vacuole-free nickel particle of 9–14 μ in longest diameter in its protoplasm was introduced into a glass capillary of 70–100 μ in diameter, so as to make the organism take monopodal form. The capillary was mounted so that

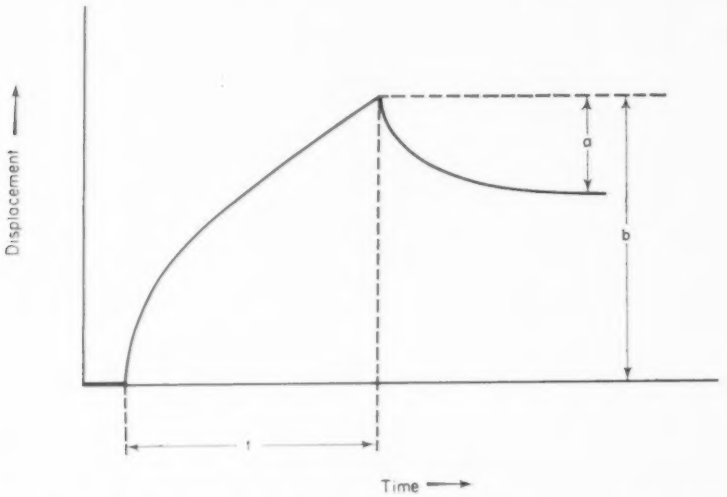

FIG. 7. Diagrammatical representation of the photographic record as is shown in Fig. 6. *a* is the total displacement by magnetic force applied for *t* seconds. *b* is elastic return.

the amoeba moved in a horizontal plane and perpendicularly to the direction of magnetic field. In this way the protoplasmic nickel particle in the amoeba could be moved sideways by magnetic force at a right angle to the direction of flow. The track of the particle was recorded on a sliding photographic plate by projecting the microscopic image of the particle through a slit. The movement of a nickel particle within the ectoplasmic gel pulled by magnetic force applied for a limited period is illustrated in Fig. 6. As is shown in the figure, the nickel particle invariably showed a rapid movement followed by a gradual shift under the continued influence of magnetic force. As soon as the current through the electromagnet was cut off, the particle returned for some distance, partly resuming its original position. The nickel particles suspended in glycerol solution, which is a Newtonian fluid, always moved with a uniform velocity during application of magnetic force and never reversed when magnetic traction was stopped. Therefore, the above-mentioned movement of nickel particles within ectoplasm does not seem to be due to a residual magnetic field. This result may indicate that the ectoplasmic gel is a viscoelastic colloid. To facilitate the analysis of this observation, the ratio of the elastic return

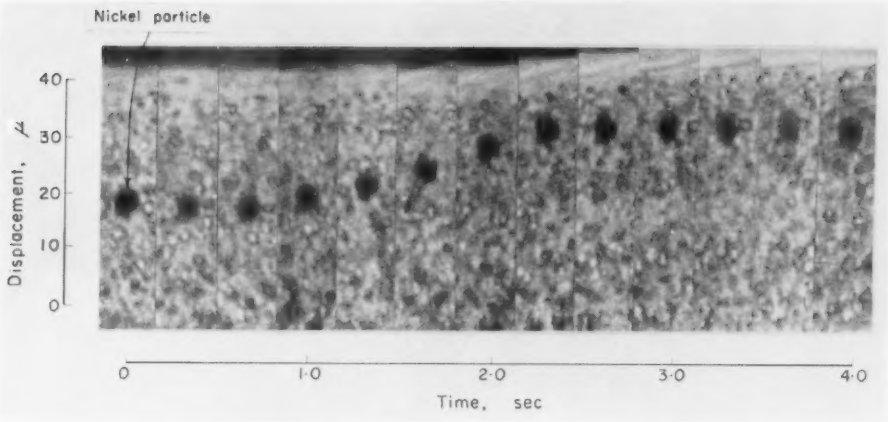

FIG. 1. Microphotographs of nickel particle within endoplasm. Each successive strip was taken at an interval of $\frac{1}{3}$ sec. During application of the magnetic force, the particle shows displacement with uniform velocity and after its removal no elastic return is observed.

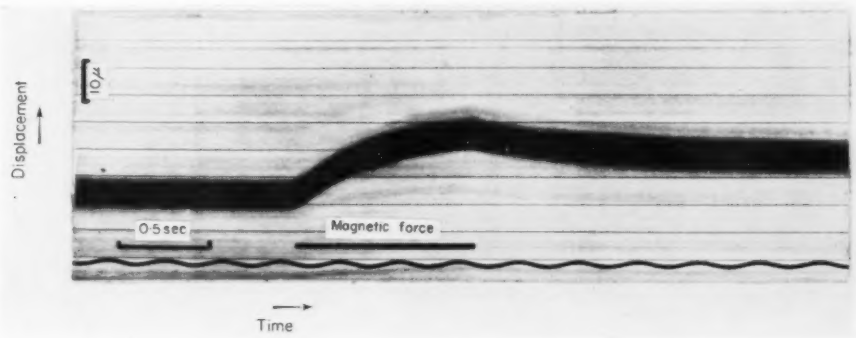

FIG. 6. A record of movement of a nickel particle within the ectoplasmic gel by magnetic force.

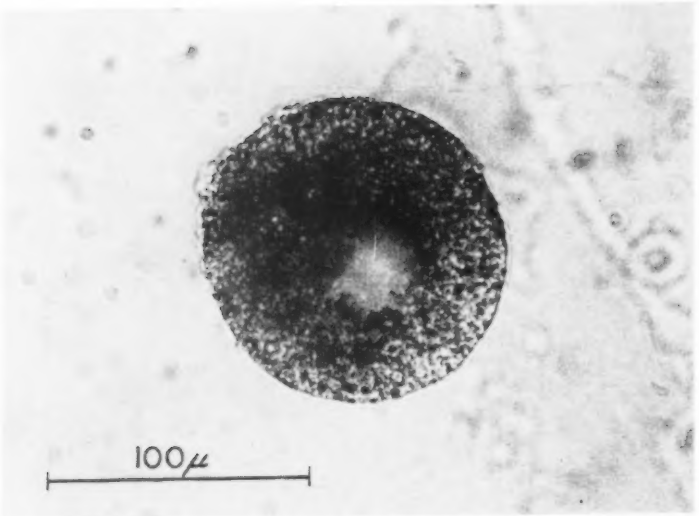

FIG. 12. Amoeba taking a spherical shape after the application of mechanical stimuli.

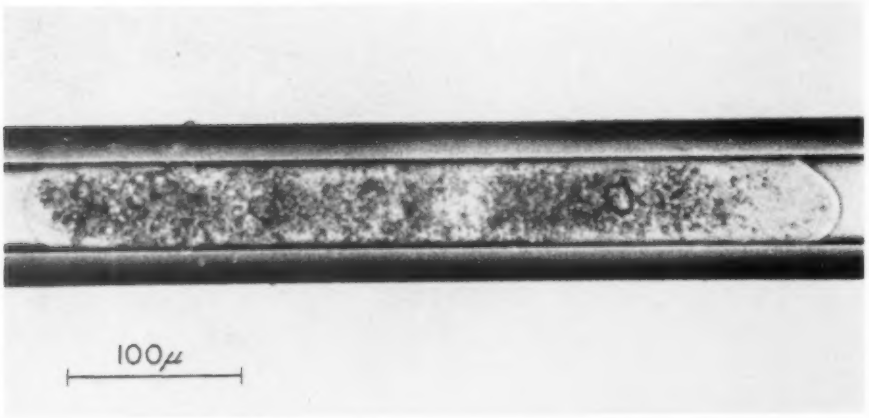

FIG. 13. Amoeba introduced into a glass capillary.

(*b*) to the whole displacement of the nickel particle (*a*) was taken to represent the elastic nature of the system (Fig. 7). The elasticity values were plotted against the position of the measured site along the anteroposterior axis of ectoplasmic gel (Fig. 8). The result revealed an anteroposterior gradient in which the greatest elasticity of ectoplasmic gel is in the tail and the smallest is in the front part of the moving amoeba.

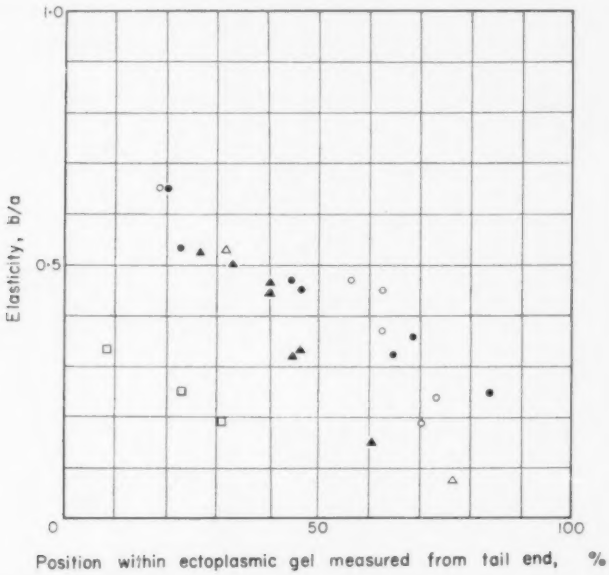

FIG. 8. Anteroposterior difference of elasticity within the ectoplasmic gel. Each symbol represents the result obtained from a single specimen.

The frictional resistance due to the viscosity of the protoplasm should be inversely proportional to the moving velocity $[(a-b)/t]$, for the magnitudes of the applied magnetic force were constant in successive measurements on the same specimen. Consequently, in the present analysis $[t/(a-b)]$ was taken to represent the viscosity value. These viscosity values plotted against the position of the measured site along the long axis of ectoplasmic gel are shown in Fig. 9. Again a gradient is seen in which the ectoplasmic gel of the posterior region shows a tendency to be more viscous than that of anterior region.

Since the local ectoplasmic elasticity and viscosity can be measured simultaneously from a single record, it should be possible to learn to what degree these two values are correlated with each other. As is shown in Fig. 10, there is a distinct relationship in which elasticity and viscosity of the ectoplasmic gel are positively correlated.

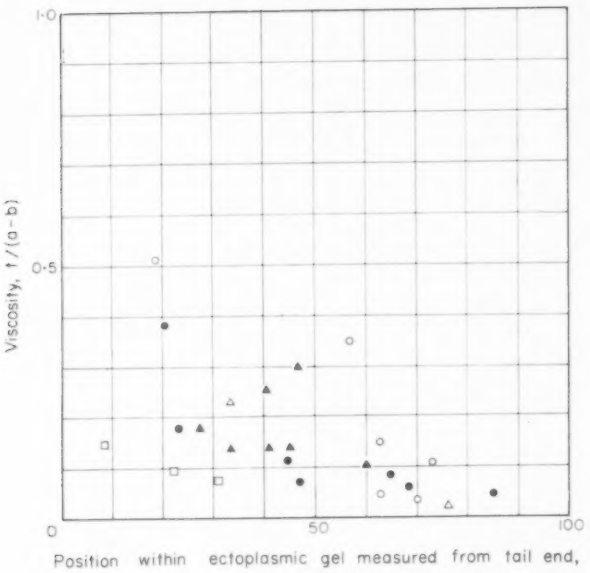

FIG. 9. Anteroposterior gradient of viscosity within the ectoplasmic gel. Each symbol represents the result obtained from a single specimen.

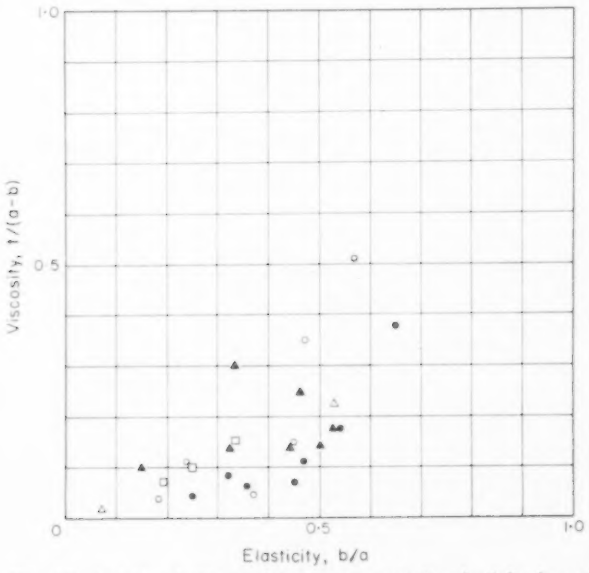

FIG. 10. Relation between the viscosity and the elasticity in each simultaneous measurement.

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In the most simple viscoelastic model which consists of a pure viscosity and pure elasticity connected in series, the apparent deformation by a constant force may be taken to be represented by the sum of elastic deformation and plastic deformation due to viscosity. Therefore, if this were the case in *Amoeba* ectoplasm, the nickel particle might be expected to move as is shown in Fig. 11, in which *AO* or *CD* represent the pure elasticity and the inclination of line *OD* or

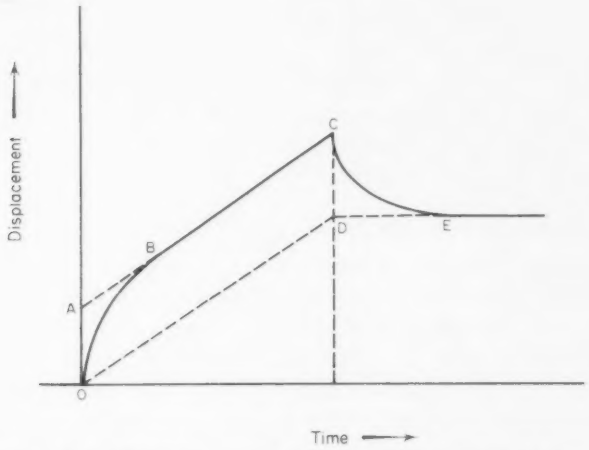

FIG. 11. Diagram showing the movement of particle in the simplest rheological model in which pure elastic and pure viscous elements lie in series.

BC represent the pure viscosity. However, the actual trace (Fig. 6) of the movement of a nickel particle within the ectoplasmic gel indicated that the later part of the line during the pulling is not straight as is shown in Fig. 11, but is always convex toward the time axis. This fact seems to suggest that there is a retarded elasticity within the ectoplasmic gel of *Amoeba*.

(4) *Contraction of ectoplasmic gel*

It was observed that in moving amoebae the distance between any pair of granules within any part of the ectoplasmic gel became progressively shorter. The shortening occurred quite irrespective of the relative directions in which the two granules lay. Therefore, three-dimensional contraction, or volume-decrease seems to occur in the ectoplasmic gel of moving amoebae. On the contrary, the distance between granule pairs within the ectoplasm did not change when protoplasmic streaming was stopped. If the degree of such a contraction within ectoplasmic gel per unit time was measured at the anterior, middle and posterior parts of moving amoeba in order to study the nature of ectoplasmic contraction, the rate of contraction in the posterior region was found to be clearly higher than in the other parts (Table 3).

The stochastic significance of this observation was confirmed by the *t*-test. With regard to the difference in rates between posterior and anterior parts, and that between the posterior and middle parts, the values of *t* were estimated as

TABLE 3—DECREASE OF DISTANCE BETWEEN TWO GRANULES WITHIN ECTOPLASMIC GEL IN MOVING AMOEBA, EXPRESSED AS PER CENT OF TOTAL INITIAL DISTANCE PER SECOND

	Posterior	Middle	Anterior
No. of measurements	99	84	83
Mean value ± S.D.	5·4 ± 2·1	1·8 ± 0·91	2·6 ± 1·2

11·1 and 33·3 respectively. Therefore, the rate of contraction in the tail region may be higher than in the other two parts, with a reliability of more than 99·9 per cent, which corresponds to the *t*-value of 3·29.

(5) *Cell volume and colloidal state of protoplasm*

It has been pointed out by Pantin (1923) that ectoplasmic contraction resulting from syneresis takes place at the rear part of moving amoebae. Allen & Roslansky (1958) have proven the existence of an anteroposterior gradient of water content within the cytoplasm. The present experiments conform fairly well with these earlier observations since we found that the ectoplasmic gel of moving amoebae exhibits a volume decrease and higher visco-elasticity at the posterior part. Such anteroposterior gradients of contractility and viscoelasticity of ectoplasm may be due to differences in water content (Allen & Roslansky, 1958).

The observed correlation between increased viscoelasticity values and three-dimensional contraction in the ectoplasmic gel suggests that the decrease of visco-elasticity due to solation should inevitably accompany the volume increase of the protoplasmic colloid. Whether the volume of the living protoplasmic colloid is larger in the sol state than in the gel state, was tested in the following experiment.

As material for this test a very actively growing culture was selected. When repeated mechanical stimuli, such as given by prodding or by water jet produced by a minute glass pipette, were applied to amoebae, the pseudopodia always retracted and the organism became dark and usually took a spherical shape with a smooth surface (Fig. 12). Crushing the amoeba in such a state by means of a micro-needle proved that the entire protoplasm was in the gel state. This fact was also confirmed by the horizontal observation of the spherical cell exhibiting no deformation under the influence of gravity. The volume of the cell in this spherical state was measured. (Table 4.) The range of error in this calculation was less than 14 per cent. Then the spherical amoeba was transferred to a watch glass containing eosin solution of 10^{-5} per cent in inorganic culture solution. In this the amoebae resumed active movement under strong illumination. Within 20 or 30 sec a number of very minute pseudopodia were extruded from part to part on the spherical cell surface. A few of them were particularly extended and later became the principal pseudopodia in amoeboid movement. At that time the protoplasm in

the central part of the spherical cell began to stream. It took several minutes before the amoeba showed the normal locomotion of the proteus type again. Then the amoeba was introduced into a glass capillary of 52·6 μ in inner diameter by negative hydrostatic pressure of a 3–8 cm water column. As is shown in Fig. 13, the organism was in close contact with the capillary wall and was forced to take a cylindrical form, and it showed well-marked endoplasmic streaming. The whole length of the cell and the diameter of streaming endoplasm were measured. The total volume of the cylindrical cell was calculated (Table 4). Assuming a uniform

TABLE 4—CELL VOLUME AND COLLOIDAL STATE OF AMOEBA PROTOPLASM

Number of amoeba	Volume of spherical amoeba in gel state (10^{-7}.cm^3)	Volume of moving amoeba (10^{-7}.cm^3)			Volume of spherical amoeba after recovery (10^{-7}.cm^3)	Ratio† $\frac{V_2}{V_1}$
		Total	Sol	Gel		
1	9·9 ± 1·1	15·8 ± 0·27	5·6	10·6	10·6 ± 1·4	1·60
2	9·1 ± 0·87	13·8 ± 0·29	4·6	9·2	8·7 ± 1·0	1·59
3	7·9 ± 0·95	11·4 ± 0·39	4·2	7·2	7·1 ± 0·85	1·61
4	6·8 ± 0·86	10·5 ± 0·33	5·1	5·4	6·5 ± 0·87	1·62
5	7·0 ± 0·60	10·4 ± 0·29	2·7	7·7	6·8 ± 0·86	1·53
6	6·2 ± 0·81	10·0 ± 0·25	3·3	6·7	6·8 ± 0·86	1·61
7	5·3 ± 0·73	8·4 ± 0·25	2·8	5·6	5·3 ± 0·73	1·58
8	11·4 ± 1·2	14·4 ± 0·29	2·7	11·7	9·8 ± 1·1	1·47
9	9·9 ± 0·4	13·4 ± 0·29	4·3	8·8	8·7 ± 1·0	1·54
10	15·6 ± 0·54	25·2 ± 0·29	7·6	17·6	*	1·61
11	7·0 ± 0·32	10·4 ± 0·33	3·4	7·0	*	1·49
12	9·9 ± 0·40	15·3 ± 0·56	4·0	11·3	*	1·55
13	6·4 ± 0·30	10·1 ± 0·22	3·3	6·8	*	1·58
14	6·8 ± 0·86	11·1 ± 0·29	3·5	7·6	*	1·63
					Mean	1·57

* The data on those amoeba in which smooth spherical shape was never resumed are lacking.
† V_1 and V_2 represent the volume of spherical amoeba and that of monopodal one respectively.

thickness of endoplasmic sol over the whole length, the volume of endoplasm was calculated as a cylinder with a diameter measured at the middle part. The volume of ectoplasmic gel was expressed by the difference between the volumes of whole cell and of endoplasmic sol. After such measurement of monopodal amoebae was complete, the organism was carefully taken out of the capillary and it was returned to the watch glass filled with culture solution. The animal usually recovered the normal *proteus* type locomotion within a few minutes. By applying repeated mechanical stimuli similar to those given before the first measurement, the cell was made spherical again and the final volume was measured with the result shown in Table 4.

A remarkable increase in protoplasmic volume of the amoebae was found, when the shape of the cell or the colloidal state of protoplasm changed from spherical or gel state to cylindrical or sol state, respectively. Furthermore, in some abnormal

amoebae in which the protoplasm was entirely solated, and no protoplasmic streaming was observed, the cell volume was as much as twice as large as in the spherical state. The volume of gel part of moving amoebae was usually larger than the entire cellular volume of spherical amoebae. Such an increase in volume of ectoplasmic gel, associated with the decrease of viscoelasticity in moving amoebae, compared to spherical ones, may be due to an increase in water content. It has already been pointed out that there is an analogous positive correlation between volume, colloidal state and water content of ectoplasmic gel in single monopodal amoebae.

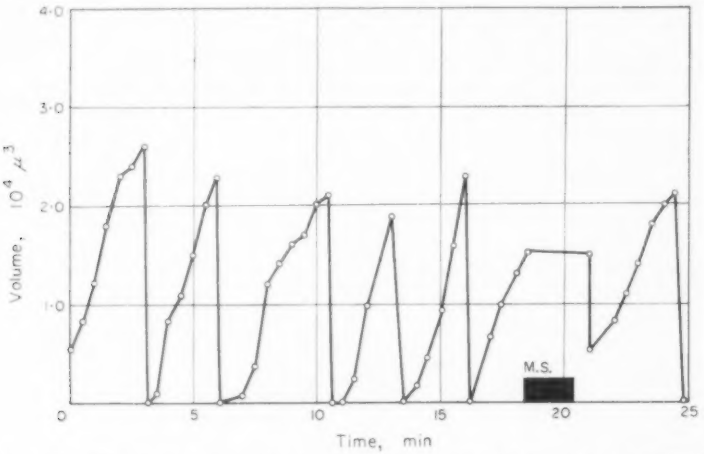

FIG. 14. The effects of the mechanical stimulation on the growth and pulsation of contractile vacuole. M.S. shows the duration of stimulation.

Accordingly, it has become clear that the amoeba was dehydrated during mechanical stimulation which caused rounding up and gelation. Since the contractile vacuole is known to be osmoregulatory (Kitching, 1938), the relation of protoplasmic gelation induced by the stimulation to elimination of water by the vacuole seemed to be worth studying. In such experiment an amoeba was mounted in a hanging drop of culture solution in a moist chamber, and the diameters of the contractile vacuoles were measured successively. After unison of several minute original vacuoles into a large one which usually occurred a few minutes after the first appearance, mechanical stimulation was given to the animal by prodding and rubbing by means of a glass needle, which caused the animal to take a more or less rounded shape. It could be ascertained that during the stimulation the pulsation of the vacuole never took place, and the vacuole exhibited little, if any, expansion (Fig. 14). Therefore, it may be concluded that the elimination of water during protoplasmic gelation is not directly related with the activity of contractile vacuole. It is more likely that water may be exchanged directly through the cell surface.

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DISCUSSION

The first recorded use of magnetic method in the study of protoplasmic viscosity appeared in the classical paper of Heilbronn (1922), who inserted small pieces of iron into slime moulds. It appears that whatever else was being measured, it was certainly not the viscosity. In order to estimate viscosity it is necessary to measure a rate of movement, whereas Heilbronn determined the magnetic field that would be just enough to move the particle. Using tissue-cultured fibroblast cells, Crick & Hughes (1950) conducted a careful experiment in which an ingested iron rod was twisted by magnetic force. They proved the existence of cytoplasmic viscoelasticity. However, as to the actual value of this viscosity they could only make a rough estimation of the order of 10 poises. In the present study more exact measurements on protoplasmic viscosity and viscoelasticity were made by drawing the nickel particle within amoeba protoplasm.

The first experiment showed that amoeba endoplasm may be a purely viscous fluid. Howard (1932) came to this conclusion using sea-urchin eggs in a centrifuge method. Although Howard proposed that the protoplasm of sea-urchin eggs might behave as a Newtonian fluid, it has been proven in the various other cells that protoplasm is a non-Newtonian or viscoelastic fluid (Seifriz, 1924; Pfeiffer, 1937; Norris, 1940; Crick & Hughes, 1950). Accordingly, it is conceivable that the elasticity of amoeba endoplasm, if it exists, may be too small to be detected by the present method.

A wide range in endoplasmic viscosity of 0·09–3 poise has been found in the studies summarized here. Measuring the rate of Brownian movement of granules, Frederikse (1932) obtained the value of 0·01–0·3 poise for the endoplasmic viscosity of *Amoeba verrucosa*. On the other hand Heilbrunn (1926) estimated a value of twice that of water in *Amoeba dubia* by a centrifuge method. The latter value is much lower than those obtained by Frederikse or those yielded by the experiments reported here. It seems probable that thixotropy within the endoplasm due to the drastic treatment of centrifugation might have caused the observed low viscosity values. Since the value of endoplasmic viscosity showed considerable variation irrespective of constancy of the magnetic force, it is quite probable that the variability of the measured viscosity is not due to any artificial factor but indicates some active and probably local difference in colloidal state of endoplasm.

Assuming that the endoplasm flows passively through the ectoplasmic tube of a uniform thickness, the pressure difference at both ends of endoplasmic streaming was calculated to be about 30–115 dyn/cm^2. These values are higher than those calculated by Heilbrunn (1952). On the other hand a value as high as a 10 cm water column was measured by Kamiya (1940) as the maximal force generated by slime mould plasmodium. However, it is questionable whether or not the protoplasm could produce such a large force during usual streaming. Recently Allen & Roslansky (1959) sucked the endoplasm of *Chaos chaos* into a narrow glass capillary by negative hydrostatic pressure and estimated the value of $5{\cdot}6 \times 10^3$ dyn/cm^3 for the pressure gradient enough to induce endoplasmic streaming. Their value is somewhat higher than the range of $8{\cdot}3 \times 10^2$–$3{\cdot}3 \times 10^3$ dyn/cm^3 calculated

from the present data described in Table 2. Allen & Roslansky must have also measured the maximal force which protoplasm could exert counteracting the external hydrostatic pressure. The author occasionally observed that the amoeba in a capillary showed outward streaming of endoplasm and escaped from the capillary against a negative hydrostatic pressure of a water column of several centimetres.

While the streaming velocity of endoplasm showed a wide variation, the calculated values of pressure difference at both ends of streaming were relatively steady in single amoeba (Table 1). It is accordingly conceivable that the local change in endoplasmic viscosity does in fact play an important role in causing endoplasmic streaming.

As to the local viscoelasticity of the ectoplasmic gel, the experiments of Section (3) reveal the existence of anteroposterior gradient. Both viscosity and elasticity are higher in the posterior than in the anterior parts in moving amoebae. The experiments of Section (4) suggest that a three-dimensional contraction (i.e. the volume decrease) may occur in the ectoplasmic gel in moving amoebae; the velocity of the contraction is significantly higher in posterior regions than in other parts. Allen & Roslansky (1958) have reported an anteroposterior gradient of water content within the cytoplasm in moving amoeba. These results conform to a remarkable degree, and together they may be taken as evidence for that a caudad shift of a part of the ectoplasmic gel as amoebae progress may lead to a three-dimensional contraction, or volume decrease, accompanying the loss of water and of an increase in viscoelasticity values.

The experiments described in Section (5) apparently indicate that the protoplasm undergoes a remarkable increase in volume when it is transformed from gel to sol. Therefore, it may be assumed that during solation amoeba protoplasm may regain the volume lost in ectoplasmic contraction. Marsland & Brown (1942) have suggested that the volume increase might take place during gelation of amoebae.

The mechanical stimuli which caused the protoplasmic gelation and volume decrease as mentioned above never accelerated the activity of contractile vacuole but, on the contrary, seemed to inhibit the elimination of water through the vacuole. Accordingly, it is likely that during gelation water is not eliminated through the vacuole but directly through the protoplasmic membrane. Fowler (1941) similarly concluded that during the change in cell volume caused by a change in external hydrogen-ion concentration water is eliminated directly through the cell surface of amoebae.

Many studies and theories on the mechanism of amoeboid movement have appeared over a period of years. The proposed theories can be classified into two groups; one is based upon the ectoplasmic contraction hypothesis and the other upon the diffusion drag force hypothesis. The first theory was first suggested by Pantin (1923) and the model based for this theory was proposed by Goldacre & Lorch (1950). Although this theory appears to provide the most satisfactory theoretical interpretation of amoeboid movement, it is criticized in recent papers

by Allen (1955) and by Stewart & Stewart (1959). On the other hand, Stewart & Stewart (1959), in their diffusion drag force theory, proposed that endoplasmic streaming might be caused by the force due to the concentration gradient of some substance within the cell. However, both theories seem to be incapable of explaining satisfactorily the following observations of living amoebae:

(1) A number of small pseudopodia can be protruded from spherical amoeba in gel state with no change in cell diameter.

(2) From the side wall of the ectoplasmic tube in normally moving amoebae a small pseudopodium could be newly protruded without forming a connexion with the main flow of endoplasm. The solated area of this part of the ectoplasm became wider externally and internally and eventually it connected with the main streaming endoplasm as the new pseudopodium was continuously growing.

(3) Occasionally the endoplasmic flow stops. When it resumes, it may flow in the same direction as before or in the opposite direction. In such cases, neither initiation nor the stoppage of endoplasmic flow usually takes place simultaneously in the whole mass of endoplasm, but a wave of stoppage or initiation of endoplasmic flow could be observed to travel along the ectoplasmic tube. That is, when the advancing tip of the amoeba stops, the endoplasm continues to stream for a short time. The endoplasm ceases its streaming gradually from part to part along the pseudopodium. In the initiation of flow a portion of stopped endoplasm at any level of a pseudopodium begins to move, and then the flowing becomes progressively extended in a wave (c.f. Stewart & Stewart, 1959).

(4) Small complicated movements of granules in certain areas of stopped endoplasm could be seen before the general flow was initiated.

These observations cannot be sufficiently well interpreted by the two "classical" theories. From the data obtained in the present study the following further considerations may be offered in explanation of amoeboid movement. At the anterior region newly arrived endoplasm undergoes gelation, accompanying dehydration and simultaneous volume decrease. The water may be squeezed out into the hyaline cap and from there it would be effused out at intervals and abruptly through the cell surface. At the posterior region, after an extreme decrease in volume, the ectoplasmic gel would suddenly imbibe water through the membrane and break down to the sol state. The sol might be forced to flow forward because the volume of the sol is increased while the volume of the ectoplasmic enveloping tube is reduced. It must be admitted that imbibition of water and volume increase may be caused by the hydration of colloid particles within protoplasm. It is reasonable to conclude that the volume increase accompanied by solation or by a decrease of viscoelasticity would cause the protoplasm to flow. If this conception is valid, then the observed four unreconciled facts listed above can be interpreted as due to local solation or a local change in physical properties of protoplasm.

It has been reported by Kriszat (1950) that adenosine triphosphate (ATP) induces an increase in protoplasmic viscosity in *Chaos chaos* and in myxomyosin, which is an ATP-sensitive protein extracted from slime mould plasmodium (Loewy, 1952). Although Zimmerman *et al.* (1958) emphasized the importance of

this fact in amoeboid movement, Ts'o *et al.* (1956) showed that whether the energy liberated from ATP induces solation or gelation of myxomyosin depends upon its initial state. Furthermore, Goldacre & Lorch and Ts'o *et al.* ascertained that ATP injected into amoeboid cells causes the solation of the plasma gel adjacent to the injection needle, and it accelerates the endoplasmic streaming for a period. From these facts it may be supposed reasonably that the energy liberated from ATP may be required for developing the imbibing force in solation, which would be converted to the motive force for protoplasmic streaming.

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THE NON-IDENTITY OF THE TRANSMITTER SUBSTANCE OF CRUSTACEAN INHIBITORY NEURONS AND GAMMA-AMINOBUTYRIC ACID

E. FLOREY and D. D. CHAPMAN

Department of Zoology, University of Washington, Seattle 5, Washington

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Abstract—1. Chromatographic analysis of extracts of peripheral and central nervous system of crabs demonstrated the presence of a single ninhydrin-positive inhibitory substance which is shown to be different from gamma-aminobutyric acid.

2. Manometric determination of glutamic acid decarboxylase in crab peripheral nerve tissue gave negative results.

3. Thiosemicarbazide injected into crayfish does not produce seizures.

4. The pharmacological actions of the crustacean inhibitory substance on crustacean organs are similar to those of gamma-aminobutyric acid and of vertebrate Factor I.

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INTRODUCTION

FACTOR I has been defined as a substance present in mammalian brain which inhibits impulse generation in the slow-adapting sensory neuron of the abdominal stretch receptor organs of crayfish (Florey, 1954a). In their attempt to isolate the principle responsible for Factor I activity, Bazemore *et al.* (1957) isolated a substance identical with gamma-amino-butyric acid (GABA). These workers showed that the GABA content of mammalian brain may account for most of the Factor I activity of brain extracts.

Factor I has been proposed as an inhibitory transmitter substance in mammals by Florey & McLennan (1955), and in Crustacea by Florey (1954a, 1956, 1957). Edwards & Kuffler (1959) found that the action of GABA on the crayfish stretch receptor neurons imitates that of the natural transmitter and that it is the most potent of a number of analogous and homologous compounds tested. However, in mammalian and echinoderm preparations, qualitative differences have been found between the activity of Factor I preparations and that of GABA (McLennan, 1957; Florey & McLennan, 1959) and McLennan (1959) found that Factor I prepared according to Florey & McLennan (1955) may contain little or no GABA. According to Curtis *et al.* (1959), Purpura *et al.* (1959) and McLennan (1957), it is very unlikely that GABA is an inhibitory transmitter in mammals.

It has been shown that extracts of crab thoracic ganglia have an inhibitory activity on the crayfish heart (Florey, 1954a) and that Factor I occurs in crab peripheral nerve, exclusively in the inhibitory axons (Florey & Biederman, 1960). It, therefore, seemed of importance to decide whether GABA might account for

the Factor I activity of crustacean nerve extracts, particularly since the actions of GABA and of these nerve extracts show no qualitative differences in the crayfish stretch receptor preparation (see also Bazemore *et al.*, 1957; and Edwards & Kuffler, 1959).

METHODS

Nerve tissue was obtained from *Cancer magister* (Dana) and *Homarus americanus* Milne-Edwards. Peripheral nerves were used almost exclusively. After dissection from the live animal the tissue was weighed rapidly, added to a small volume of boiling distilled water and heated for at least 1 min. The tissue and water were subsequently homogenized and samples of the resulting extract, made up to a known volume, were taken for assay on the crayfish stretch receptor preparation and for chromatography. In later experiments the nerve tissue was directly homogenized with methanol. After centrifugation the clear supernatant was used for chromatography.

Bioassays for Factor I were carried out as described by Elliott & Florey (1956) using stretch receptors prepared from *Pacifastacus leniusculus* (Dana) and *Orconectes virilis* (Hagen). The effects of extracts and of eluates of chromatograms were also tested on crayfish heart and intestine as described elsewhere (Florey, 1954a, b, 1960c).

Chromatography was carried out on washed (0·1 N NaOH, 0·1 N HCl, H_2O in sequence) Whatman No. 1 filter paper, with butanol–acetic acid–water (50:11:25) and propanol–water (70:30) as the descending solvents.

Before application to the paper, the aqueous tissue extracts were electrodialysed (Katz & Chaikoff, 1954) to eliminate salts. This could be achieved without loss of activity. The extracts were applied to the paper in narrow streaks. After development, edge strips of the chromatograms were sprayed with a solution of ninhydrin in acetone. The remainder of the paper was cut into bands in order to separate the various ninhydrin positive areas. The bands were eluted with water, the eluates taken to dryness, and the resulting residues were dissolved in buffered saline medium (Elliott & Florey, 1956) and tested for Factor I activity. After the R_f value of the inhibitory substance was ascertained the eluate of the corresponding area of further chromatograms was applied to other papers and rechromatographed in another solvent system.

In several experiments, known amino acids (including GABA, beta-alanine, glutamic acid and L-alanine) were applied to the paper as streaks partially overlapping the region to which the nerve extract was applied.

Following the manometric method of Roberts & Frankel (1951) and Killam & Bain (1957) we determined the glutamic acid decarboxylase activity of crab nerve homogenates using rat brains as controls. Into each reaction vessel we placed 1·8 ml of a 20 per cent nerve homogenate in buffer (0·05 M potassium-acid phosphate, pH 5·9); 0·1 ml of 0·1 M NaF, 0·4 ml of 0·7 M glutamic acid and 0·2 ml of buffer containing 0·25 mg of pyridoxal phosphate were placed in side arm No. 1; 0·3 ml of H_2SO_4 were added to sidearm No. 2.

RESULTS

Bioassay for Factor I activity with the stretch receptor preparation was complicated by the presence of excitatory material in the extracts. Thus, nerve extracts containing less than 20 mg of tissue per ml rarely caused complete block of impulse

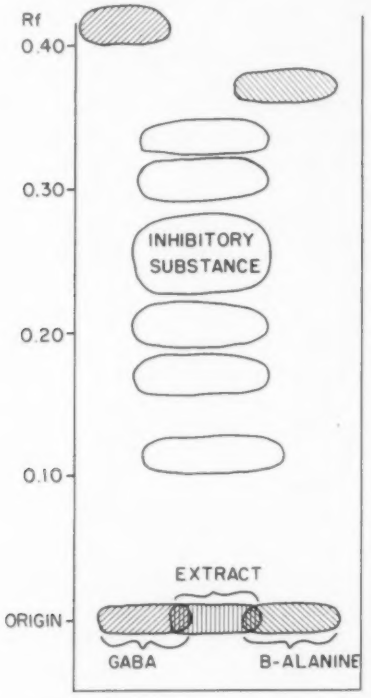

FIG. 1. Position of ninhydrin positive areas on a chromatogram of partially overlapping streaks of gamma-aminobutyric acid (GABA), extract of peripheral nerves of *Cancer magister*, and beta-alanine. Solvent: Butanol–acetic acid–water (50:11:25). Chromatograms obtained with peripheral nerve extract from *Homarus americanus* are practically indistinguishable from the one shown. Inhibitory activity is obtained exclusively from the area marked "inhibitory substance".

generation for as long as 10 sec. On the basis of several assays we estimate the inhibitory activity present in extract of 1 g of crab nerve to be equivalent to that of 90–150/μg GABA (i.e. 30–50 c.u.r., Elliott & Florey, 1956).

However, after chromatography, the effect of the excitatory material was removed and we could recover from the chromatogram inhibitory activity which, if referred to as GABA, would require the presence of up to 4000 μg of this substance per gram of fresh nerve tissue.

All of the detectable inhibitory activity present on the chromatograms could be eluted from a ninhydrin-positive band distinctly separate from that band in which GABA should have been found (see Fig. 1). We have never been able to

elute detectable activity from the area of the chromatogram to which GABA should migrate. Indeed, no ninhydrin-positive material is present in this area (detection limit 3μg) in chromatograms containing inhibitory activity equivalent to that of 50μg GABA.

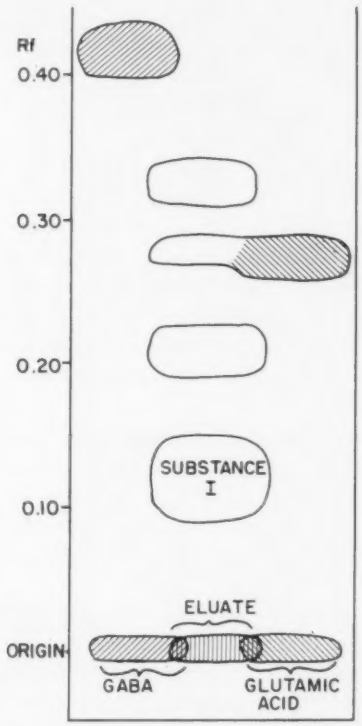

FIG. 2. Position of ninhydrin positive spots on a chromatogram of partially overlapping streaks of gamma-aminobutyric acid (GABA), eluate of the area containing the inhibitory activity obtained from peripheral nerve extract of *Homarus americanus* in a chromatogram developed with butanol–acetic acid–water, and glutamic acid. Solvent: propanol : water (70:30). All inhibitory activity was found in the area marked "substance I".

The band containing the inhibitory material was eluted from chromatograms developed with butanol–acetic acid–water and was rechromatographed with propanol–water. The presence of several substances was revealed by this procedure; however, all of the inhibitory activity was found in a single ninhydrin-positive band (Fig. 2).

In contrast to results obtained with crab nerve extracts, we have found that Factor I prepared according to Florey & McLennan (1955) from beef brain contains amounts of GABA sufficient to account for most of the inhibitory activity when tested with the stretch receptor technique. A second ninhydrin-positive

inhibitory substance was, however, detected in the chromatograms (butanol–acetic acid–water). Its R_f value corresponded closely to that of the inhibitory substance of crustacean nerve tissue and appears to be identical with McLennan's Fraction A of Factor I (McLennan, 1959).

The ninhydrin-positive inhibitory substance of crab nerve extracts was applied to the crayfish heart and intestine. In both organs it stops the spontaneously occurring contractions and blocks the action of acetylcholine (a more detailed account of these effects will be published separately).

We have repeatedly ascertained that picrotoxin (10^{-5} g/ml) blocks the action (on stretch receptor, heart and intestine) of the inhibitory agent present in crustacean nerve extract. In this regard this behaves in a manner similar to that of both GABA and mammalian Factor I (see Elliott & Florey, 1956).

In our chromatograms of crab nerve extracts we were able to detect considerable amounts of glutamic acid (R_f value, color of ninhydrin reaction, excitatory effect on stretch receptor, inhibitory action on heart and intestine). Since glutamic acid is the precursor of GABA in mammalian nerve tissue (Roberts & Frankel, 1951) it was surprising to find no detectable quantities of GABA in the extracts. It appeared, therefore, of importance to discover whether crab nerves contain glutamic acid decarboxylase.

We found absolutely no sign of glutamic acid decarboxylase activity in any of the crab nerve homogenates tested. Rat brain homogenates which were used for comparison in the same runs produced the expected amounts of CO_2 under the experimental conditions (287–346 μl per g tissue per hr). The absence of glutamic acid decarboxylase may well account for the absence of GABA in crab nerve tissue.

The conclusion that this enzyme does not occur in crustacean nerve tissue is supported by our finding that thiosemicarbazide has no action on the crustacean nervous system. Thiosemicarbazide is known to induce severe seizures in mammals. This compound inhibits the B_6-dependent glutamic acid decarboxylase. According to Killam *et al.* (1960) the resulting fall in free GABA in the brain is responsible for the convulsions. We injected as much as 100 mg/Kg of thiosemicarbazide into crayfish and placed these animals into water containing 0·01 mg/ml of this substance, renewing the solution every 12 hr and repeating the injections daily. In none of the eight animals studied did we observe abnormal reactions during 5 days of treatment.

In two of the animals treated with thiosemicarbazide the heart and thoracic cavity were exposed and perfused (Florey, 1957) with a solution of 0·01 mg thiosemicarbazide per ml. In normal crayfish stimulation of either the left or right cardio-inhibitory nerve with 30–40 pulses per second leads to complete diastolic arrest (Wiersma & Novitski, 1942; Florey, 1960a). The same was found with the thiosemicarbazide-treated animals. The inhibitory nerves, as in normal animals, remained functional for several hours. These results indicate that the production of inhibitory transmitter does not involve the action of a thiosemicarbazide-sensitive decarboxylase.

It is striking that in those organisms in which it does not occur, GABA imitates the naturally occurring inhibitory transmitter (Kuffler & Edwards, 1958) and that in those animals in which it is found, its actions differ from that of the transmitter.

From our data it appears likely that McLennan's Fraction A of Factor I is identical with the inhibitory substance present in crustacean nerve extracts. Following an earlier suggestion (Florey, 1960b) we would like to name this inhibitory compound Substance I until identification of the compound is achieved. In accordance with the evidence presented elsewhere (Florey, 1956, 1957, 1960b; Florey & Biederman, 1960) Substance I may very well be the transmitter substance of inhibitory neurons of decapod crustacea. We recognize, however, that the chemical evidence is still preliminary.

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THE WATER RESERVES OF HATCHLING LOCUSTS

R. E. BLACKITH

Zoology Department, Imperial College Field Station, Sunninghill, Ascot, Berks.

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Abstract—(1) The demand for water reserves and conservation which various environments make on the locust species hatching into them is reflected by the greater efficacy of the waterproofing of the Desert locust as compared with the Red locust, which hatches into swampy grassland.

(2) This difference persists even when the water-proofing layer of the cuticle is disrupted, and abrasion is probably important to locust hatchlings exposed to blown sand or dust; the vermiform sheath protects them during the ascent through the soil, after eclosion.

(3) Water conservation is enhanced by the lysis of proteins to augment the osmotic pressure of the blood of starving, dehydrated hatchlings. This process is rapid in hatchlings whose cuticle has been abraded, and the behavioural changes which accompany dehydration in Catantopids will also tend to conserve water.

(4) Generally, the survival of hatchlings in dry atmospheres is greatest for those which weigh most at eclosion, and most of such differences of weight represent variation in the water reserves.

INTRODUCTION

THE MORTALITY of locusts and grasshoppers is often highest during the earliest instars (Richards & Waloff, 1954; Pickford, 1960). Despite the critical nature of embryonic and early post-embryonic life in acrididae, Uvarov's (1948) review of progress in locust physiology has little to say on the topic and only recently has it been shown that survival under adverse conditions is closely linked with the weight of the hatchling (Albrecht & Blackith, 1960). This variation in size is in turn intimately associated with many other physiological attributes connected with the phase status of the parent insects, and Albrecht *et al.* (1959) have suggested that the greater size of hatchlings from crowded parents represents a sacrifice of reproductive potential, as measured by the reduced numbers of ovarioles in the female, in favour of a better endowment of food reserves in the most general sense. This switch mechanism operates so that when the population density undergoes marked changes, a wide range of offspring is produced so that at least some are likely to survive any but the most oppressive conditions, since the extremes of the range are adapted to quite different environments.

This paper explores the nature of the differences in weight of hatchling locusts, and in particular the water reserves which they embody. A subsequent paper examines the biochemical differences between locust hatchlings of different sizes in more detail, especially their food reserves. These investigations are closely

integrated with the experiments of Dr. F. O. Albrecht, of the Evolution Laboratory in the Sorbonne, who is making a detailed study of the survival capacities of the different types of locust with which we are concerned.

EXPERIMENTAL

Nearly all the locusts used in this work were reared by Dr. Albrecht in Paris on a diet of *Festuca pratensis*, as described by Albrecht *et al.* (1958). They were transferred to Sunninghill either in the egg stage, or as dried corpses after Dr. Albrecht's starvation experiments had been completed, in which case the initial weights were determined by him. The three species used were the Migratory locust, *Locusta migratoria migratorioides* R. & F.; the Desert locust, *Schistocerca gregaria* (Forskål) and the Red locust, *Nomadacris septemfasciata* Serville.

Larvae issuing from the egg-pods were weighed individually in aluminium pill-boxes, killed by rapid heat-shock and dried for 1 hr at 105°C. The moisture content so determined has been checked against bulk determinations by the toluene distillation technique with good agreement. Weighings of individual insects were accurate to 50 μg, the effective limit in view of the heavy electrostatic charges accumulated during drying.

Total water-soluble solids were determined by extracting the dried larvae in a micro-Soxhlet for 90 min, drying and re-weighing. Individual larvae were placed in filter-sticks inserted in the micro-Soxhlet.

The moisture content of hatchlings in relation to their total weight

Individual analyses of forty *Locusta* larvae from one egg-pod showed that the percentage moisture content was independent of the total weight at eclosion. The analyses of individuals of the two Catantopids, however, showed that the percentage of water increased steadily as the initial weight increased. If hatchling *Schistocerca*, for example, contained on an average 79 per cent the smallest would contain some 74 per cent, the largest 84 per cent. The importance of these figures in the ecology of the hatchlings needs no emphasis when it is recalled that the first instar of all three species die when the moisture content drops to about 63 per cent so that the largest larvae have about twice as much "disposable water" as that possessed by the smallest larvae. The young instars are the most sensitive to loss of water, adult *Schistocerca* being able to tolerate a reduction to 36 per cent of water (Millot & Fontaine, 1937).

Forty larvae from each of two Red locust pods were analysed, and were found to contain 1 per cent more water for every 1 mg extra weight, as compared with the figure for the Desert locust where about 2 per cent of additional water was found to correspond to 1 mg extra weight. These regressions of water content on total weight are influenced by the moisture content of the sand in which the pods are laid, as are the average moisture contents of the larvae issuing from the pods. The absorption of water from the external sand to the egg within the pod must be a rapid process, since in one experiment the sand was watered whilst larvae were emerging slowly from rather dry sand, and both the mean water content of the emerging larvae, and

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the rate of increase of moisture content with weight, were restored to the level expected for moist sand.

The effects of loss of water on hatchling locusts

Any attempt to study the influence of dehydration on locusts by exposing them to dry atmospheres must confound these effects with those of deprivation of food and of ageing, since the first instar larvae can survive several days under such conditions. To avoid these difficulties, the cuticular waterproofing of hatchlings was disrupted with abrasive dusts, such as are used in dry climates for the control of insect infestations.

TABLE 1—RATES OF LOSS OF WATER FROM HATCHLING LOCUSTS WITH INTACT OR ABRADED CUTICLES (AT 25°C)

Species	Phase	Mean weight (mg)	Loss with intact cuticle		Loss with abraded cuticle	
			(mg/hr/larva)	(%/hr)	(mg/hr/larva)	(%/hr)
Locusta migratoria	*solitaria*	12·4	0·040	0·40	0·637	6·37
Locusta migratoria	*gregaria*	17·4	0·056	0·41	0·699	5·00
Nomadacris septemfasciata	*gregaria*	18·9	0·076	0·51	0·882	5·88
Schistocerca gregaria	*gregaria*	20·2	0·060	0·38	0·507	3·13

A number of *Nomadacris* larvae from crowded parents were allowed to emerge inside a large glass cage (approximately 2 ft × 2 ft × 1 ft). The cage was kept at 25°C and 56 per cent r.h. At no time were the insects handled, since Beament (1959) has shown how even gentle handling can falsify the results of experiments intended to investigate rates of loss of water. Of the liberated larvae, a sample of sixty was removed for immediate analysis and the water content proved to be 78·6 per cent. The cage was left undisturbed for 15 hr after which time a further sample of sixty larvae was removed and shown to have lost only 0·6 per cent of water by comparison with the previous sample. The remaining larvae were then dropped into a long-necked flask containing the abrasive dust "Neosyl", a hydrated siliceous dust with particles of about 0·1 μ; its general properties have been described by Harlow (1957). When the larvae had acquired a coating of the dust they were returned to the cage, which was illuminated with fluorescent strips several feet above it. A small sample of five larvae was used to establish the weight of dust adhering to the larvae.

After a further 3 hr, during which time the behaviour of the insects was watched continuously, the larvae were moribund and were removed for analysis.

The moisture content of these twenty-five hatchlings had dropped to 64·6 per cent after the additional 3 hr exposure with the cuticle lightly abraded by the dust. Thus abrasion increases the rate of loss of water by a factor of 11·7 (Table 1).

The findings of this experiment were confirmed by exposing Desert and Migratory locust larvae in the same sequence of treatments. Hatchlings of both phases of *Locusta* were used, in order to examine any maternal influences on the water-proofing of the progeny of crowded mothers or of mothers isolated except for the time needed to permit of copulation.

The results of these treatments are shown in Table 1 where the rates of loss of water are expressed in absolute units (mg/hr/larva) and also as a percentage of the initial content of water, so as to take account of the different sizes of the insects.

The temperature coefficient of transpiration in locust hatchlings seems to be slight; data obtained during Dr. Albrecht's experiments show that at 31°C the rate for *gregaria* hatchlings of the Red locust has increased only to 0·087 mg/hr/larva from 0·077 at 25°C. For *Locusta* hatchlings of the same phase the increase is not measurable by these techniques, Dr. Albrecht finding a rate of 0·055 mg/hr/larva at 31°C, and Hunter-Jones (1958) finding 0·049 at 32°C, as against the 0·056 mg/hr/larva at 25°C (Table 1.)

The mobilization of haemolymph solutes to augment the osmotic pressure and reduce evaporation

The determinations of the total water-soluble solids in the dried larvae showed that the percentage of such solids increased on starvation by an amount far greater than could be accounted for by the loss of water on starvation. For instance, the issue of a *gregaria* egg-pod of *Nomadacris* was divided into three equal parts each containing seventeen hatchlings in the course of Dr. Albrecht's starvation experiments. Those hatchlings which were killed on eclosion contained 29·7 per cent water-soluble solids on a dry weight basis; those starved to death at 35 per cent r.h. contained 40·0 per cent and those starved to death at saturation humidity contained 37·3 per cent. These figures were typical of very many analyses of this kind using all three species of locust and both phases.

To show that this increase is a response to dehydration rather than to the other effects of starvation, samples of Red locust hatchlings were treated with Neosyl dust as before, and the contents of water-soluble solids, initially 24·3 per cent of the dry weight of this sample, had increased to 28·9 per cent after 3 hr exposure at 25°C. A parallel sample left for over 12 hr without abrasion showed only an increase to 26·1 per cent.

Sexual dimorphism in the first instar

Although the morphological differences between the sexes in the first instar are not well developed, there seem to be differences of size associated with the position of the egg in the pod from which the larva hatches. In the Migratory

locust there is a substantial, if capricious, sexual dimorphism in terms of the total weight at eclosion, the females being, on an average, some 17 per cent heavier than the males (Albrecht *et al.*, 1958). The difference in weight was significant at the 5 per cent level in only thirteen out of the twenty-seven egg-pods analysed, although the overall difference of weight was significant at the 0·1 per cent level for the twenty-seven pods as a whole.

This matter has been taken further by analyses of the total and dry weights of the sexes of hatchling Red, Migratory and Desert locusts. The Red locusts were handled in batches of ten, six batches of males and six of females. No significant differences of either wet or dry weights were found. Similarly, when eighty males and eighty females of first instar *Locusta* were analysed in batches of ten, no differences of note were uncovered. However, the analysis of sixty male and sixty female Desert locust hatchlings in batches of ten gave clearcut differences, probably because in this species the gradients which exist within the egg-pod, and hence presumably within the maternal ovaries, are much more marked than in the other species. The mean total weight of the males was 22·33 mg and that of the females 22·83 mg; lest this difference be thought to be trivial the ranges of the total weights (means of batches of ten) are only 21·81–22·92 mg for the males and 22·48–23·24 mg for the females. The individual analyses reported above show that in this species there is a tendency for the heavier hatchlings to have the smaller weights, and conversely. This situation obtains with respect to the sexual dimorphism, there being a significant difference between the mean dry weight of the males (4·415 mg: range of mean weights of the six batches of ten, 4·20–4·58 mg) and that of the females (4·369 mg: range of batch weights 4·10–4·30 mg). It follows that the percentage moisture content of the two sexes issuing from the same pod is different, the males containing 79·66 per cent water and the females 81·15 per cent. This difference could arise from the tendency to differential fertilization along the string of embryos within the mother, but there are several critical lacunae in our knowledge of this subject.

In order to examine the nature of the difference of dry weight further, each batch of larvae was analysed for its chitin content, as a measure of the structural material in each. No significant differences were found, so that the weight differences may reside in the food reserves of the larvae.

DISCUSSION

The critical nature of the water reserves of hatchling locusts in determining their survival under adverse conditions is brought out by the many ways in which they are adapted to conserve water. In one sense this adaptation begins with the maternal response to crowding, since smaller Catantopids from isolated parents have lower reserves than have larger larvae from swarming populations. This situation has a certain plausibility since isolated locusts are rather more likely to find themselves in areas of available vegetation, whereas the progeny of swarms are likely to hatch in an environment where food has to be searched for, and water

conserved meanwhile. Rainfall during the incubation of the eggs is also of critical importance (Albrecht, 1956) and, by modifying the initial moisture content of the larvae, helps to determine their survival.

The locomotor behaviour of young locusts is instantly modified by the application of dust, which penetrates the jointed structures of the integument, making movement difficult (Harlow, 1957). But in other respects the larvae soon behave in a fashion never seen in normal hatchlings. They become strongly negatively phototropic, hiding in corners of the cage or wherever shade is available, even though the illumination is virtually devoid of heat content. They also appear to be thigmotactic, coming to rest in crevices or against other stationary larvae. Assuming that field abrasion acts in the same way as laboratory abrasion, such behaviour would tend to bring parched larvae into vegetation or other cooler, damper surroundings. Normal locust larvae tend to be slightly, positively, phototropic (Cornwell, 1955).

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The low temperature coefficient of the transpiration of locust hatchlings suggests that active as well as passive control is possible. In fact, all three of the species examined are able to mobilize water-soluble substances in the haemolymph, almost certainly amino-acids derived from the hydrolysis of proteins, to augment the osmotic pressure and hence to reduce the rates of evaporation during starvation or when the cuticle is abraded. Treherne (1953), discussing the osmotic regulation of aquatic insects, comments that many diverse species have "solved" the evolutionary problems of re-entry into water by the same general mechanism. We may rather enquire whether this mechanism is not widespread among the insects, being utilized to regulate the development of eggs imbibing water from their environment, so that its particular use by aquatic insects becomes less surprising.

Other insects are known to possess means by which large molecules dissolved in the blood may be broken down to smaller units and thereby to augment the osmotic pressure. In aquatic insects, part of whose osmotic pressure stems from chloride ions this means of regulation is elicited, as a response to loss of chloride (Wigglesworth, 1938; Ramsay, 1950). This conversion of proteins to amino-acids also occurs in the dragonfly larva *Sialis* (Beadle & Shaw, 1950) where it is reversible on the restoration of the chloride. That the retention of proteins and amino-acids in the locust haemolymph entrains the retention of excessive amounts of water has been demonstrated by Weed-Pfieffer (1945) as a consequence of the extirpation of the corpora allata, and Slifer (1934) has noted that locust eggs immersed in various Ringer solutions can regulate the egress of water better than they can regulate the ingress.

Insects such as locusts which hatch from eggs laid in damp soil, from which water has to be imbibed (Shulov & Pener, 1959) have to face problems of osmo-regulation not unlike those of aquatic insects. Laughlin (1957) followed the uptake of water by the eggs of *Phyllopertha horticolor* and he noted that, although the swollen egg contained some five times its initial amount of water, the osmotic pressure of the amniotic fluid was reduced by little more than a quarter. He went

on to suggest that the embryo could break down large molecules for this purpose. That locusts of the genus *Melanoplus* use proteinolysis to the same end can be deduced from the results of Teppert (1958) who measured the amino-acid concentrations in the developing egg, both in the embryo and outside it.

The identification of the enhanced concentration of solutes in the partially dehydrated larval haemolymph with proteinolysis rests partly on the fact that the amounts of solid material involved can scarcely be accounted for by other non-ionic blood constituents. The amounts of glycogen in locust blood are small (30–40 mg per cent in the adult, according to Howden & Kilby, 1956, 1961). More positive evidence comes from experiments briefly reported by Blackith & Albrecht (1960) in which water extractions of the dried and heated larvae are compared with those of larvae which were alive at the commencement of extraction. The substantial difference between the dry-weight percentage of water-soluble solids in the two cases suggests that the difference is made up of proteins extracted from the unheated insects but denatured, and hence not extracted when the larvae are dried and heated prior to extraction. Of course, the breakdown of blood proteins would liberate appreciable quantities of the other molecular species bound to these proteins, particularly the lipids, but nothing is known of the importance of this process in the hatchling locust.

The order of the efficacy of water-proofing of three species examined here supports the hypothesis of Albrecht (Albrecht & Blackith, 1960) to the effect that the water relations of hatchlings reflect the nature of the environment into which they are most likely to emerge. Evidently, there are no important differences between the phases of the Migratory locust. The Red locust, on the other hand, is in its first instar essentially an inhabitant of swampy grasslands, and the relative water-proofing of the species is again in accordance with the suggestion that the moisture relationships of the hatchlings of locusts reflect the environment in which they most often emerge. The Desert locust hatchlings are the best proofed, not only when the cuticle is intact, but, more particularly, after some abrasion has been suffered. The first instar of the Red locust, which often hatches into a swampy vegetation, is the least able to withstand abrasion; the xerophilic Desert locust often has to face blown sand soon after hatching, although the abrasive properties of such sand may not compare closely with those of the dust used in this work.

Many acrididae hatch from the egg surrounded by a sheath consisting mainly of chitin. This sheath is retained during the ascent of the larva through the soil, in the case of those acrididae which lay in soil, and is moulted when the larva emerges into the air. Most soil insects have the cuticle in a permanent state of abrasion through contact with the sand particles and perhaps also through adsorption of the epicuticular waxes by organic matter in the soil, as seems to occur with Collembola (Ogel, 1957). Evidently, were the larval locusts to emerge from the soil with abraded cuticles, few would survive to find succulent vegetation under adverse conditions, and whatever other function the vermiform sheath may have it seems certain that it helps to protect the hatchling from abrasion.

SUMMARY

(1) The first instar of the Desert locust loses water less rapidly than does that of the Red and Migratory locusts. This is so when the cuticles are intact, and also when they have been severely abraded, increasing the rate of loss of water tenfold.

(2) Dehydration of the Catantopid hatchlings entrains behavioural changes likely to help the insects to conserve water.

(3) The vermiform sheath protects the larva from abrasion during the ascent from the egg-pod to the soil surface.

(4) Water conservation is assisted by proteolysis in the haemolymph of starving locust hatchlings, so as to increase the osmotic pressure of the blood.

(5) The longer survival of the large unfed hatchlings at low humidities is attributable to their greater initial reserves of water.

(6) The largest Desert and Red locust hatchlings from any one egg-pod contain twice as much water available for transpiration as do the smallest.

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THE FOOD RESERVES OF HATCHLING LOCUSTS

R. E. BLACKITH and G. F. HOWDEN

Zoology Department, Imperial College Field Station, Sunninghill, Ascot, Berks.

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Abstract—(1) Hatchlings of the Red, Desert and Migratory locusts have been analysed for fat, amino-acids, sugars and chitin to uncover biochemical differences associated with enhanced survival.

(2) The distribution of fat in individual larvae is highly skew, so that even when the mean fat content is high, individuals with virtually no fat reserves at eclosion can be found, and these seem to survive as well as those with high fat contents.

(3) Maternal diet influences the fat content of the larvae.

(4) During starvation of the hatchlings, some of the sugars, which are present in much smaller quantities than in the later instars, are scarcely consumed, but the content of an unknown reducing substance which may be ascorbic acid is much depleted. This substance is also more plentifully distributed in the offspring of crowded parents than in those from isolated females. No glycogen has been detected in hatchlings.

(5) *Gregaria* larvae have larger reserves of amino acids than have *solitaria*, and threonine and glycine, in particular, are depleted during prolonged starvation. These losses, and those of ascorbic acid and fat, are roughly equal to the net loss of weight during starvation to death.

(6) The chitin content of the unfed hatchlings increases during the period of starvation so that when they die the dry weight percentage of chitin has doubled. Glucosamine, an obvious precursor, appears to be absent, and has been shown to be toxic to locusts.

(7) No differences of oxygen uptake have been detected, in respect of the phase status, sex or nutritional condition of the hatchlings.

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INTRODUCTION

THE SURVIVAL of the delicate immature stages of locusts is known to depend on their weight at eclosion among other factors. Larger hatchlings live the longest when starved under dry conditions, smaller ones live longest at high humidities (Albrecht & Blackith, 1960). Moreover, the weight at birth is primarily a matter of the moisture content of the larva, there being much less variation in the dry weights of hatchlings than in their wet weights. Within the issue from a single egg-pod, in fact, the smallest larvae may have the highest dry weight (Blackith, 1961).

There is, therefore, a *prima facie* case for supposing that survival in dry atmospheres depends on the available water reserves within the insect and its ability to conserve these reserves, whereas survival under humid conditions rests on the food reserves which the insect can muster. In an earlier paper (Blackith, 1961) the first of these propositions was examined, and we now turn to the second.

The locusts analysed in these experiments had had little opportunity to lose, and none to acquire, water; neither had they had occasion to feed. Any differences

of chemical constitution in the newly emerged insect, therefore, represent differences in the operation of the maternal ovarial cycle. Such differences may be induced in insects by a variety of environmental stimuli, of which crowding is of great theoretical and practical consequence. The maternal diet may also strongly influence the growth of insect offspring (Reynolds, 1945). The work of Wellington (1960) on tent-forming caterpillars, and of Chitty (1960) on vole populations, emphasizes that crowding induced far-reaching changes not only on the first crowded generation of organisms but also on their progeny. In locusts these effects have been traced through four successive generations (Albrecht *et al.*, 1959). The capacity to show these pervasive phase changes is associated with the presence of a particular type of sexual dimorphism in acrididae, and is likely to have a genetic basis (Blackith & Verdier, 1960); but polymorphism can be engendered by the application of environmental stimuli to breeding females, under circumstances in which the operation of selection can be shown to be incapable of accounting for the observed phenomena. These maternal effects have been shown to have a direct bearing on the survival capacity of the progeny, not only in locusts (Albrecht & Blackith, 1960) but also by Wellington (1960) in the case of his Lepidoptera. A preliminary investigation of their biochemical consequences seems overdue, since there is hardly any satisfactory information concerning the biochemistry of phase differences in locusts. Mathée's (1945) analyses of later instars showed only trivial differences, apart from those of uric acid. Such differences are at least as likely to be nutritional in origin as to reflect differences of phase status. In our analyses we have studied the distribution of fat and sugars, as obvious candidates for the energy reserves; amino-acids as being implicated in the regulation of blood osmotic pressure during starvation; and chitin as a measure of the structural material of the hatchling. Ancillary studies of the specific gravity of the haemolymph have been used to trace the extent to which the hatchling retains embryonic biochemical characteristics rather than those of the later instars. The oxygen uptake of locusts, as recorded in the literature, appeared to suggest a phase difference such that *gregaria* respire more than *solitaria*. A critical review of those earlier observations has been supplemented by some of our own.

EXPERIMENTAL

The insects were reared, as in the earlier work, by our colleague, Dr. F. O. Albrecht of the Evolution Laboratory in the Sorbonne. The pure grass diet then used was abandoned in favour of a diet of hydroponically grown wheat and bran for *Locusta migratoria migratorioides* R. & F. and hydroponic wheat and the artificial diet of Howden & Hunter-Jones (1958) for *Schistocerca gregaria* (Forskål) and *Nomadacris septemfasciata* Serville. These diets avoid seasonal fluctuations in grass quality. Their main effect is to increase the fat content of the larvae. Most of the larvae were shipped to London dried after the starvation experiments of Dr. Albrecht had been performed on them, but where living insects were required, the egg-pods were brought over in damp sand and hatched in the laboratory.

For determinations of fat, the larvae were dried at 105°C, weighed and slipped into sintered glass filter sticks inside micro-Soxhlet extractors. Light petroleum (40–60° B.R.) was used to extract them for 1½ hr, a time chosen as a compromise between the conflicting desiderata of complete extraction and the avoidance of comminution of the fragile chitinous plates under the stress of the strong electrostatic charges generated. Dust from this comminution may easily cause an apparent loss of weight in prolonged extractions, working its way through the sintered glass disc. This loss is greater if the larvae are crushed before extraction. After being dried and reweighed, the larvae were further extracted for 1½ hr with water to obtain the total water soluble constituents.

The aqueous extracts were evaporated to dryness under reduced pressure, transferred to 50 per cent alcohol solution and stored at −15°C until required for the chromatographic analyses. The insects were transferred to 5 per cent caustic potash solution and kept on a steam-bath for 10 hr. The residue was then filtered, washed in water, 5 per cent acetic acid and more water, dried and weighed. This residue is regarded as being chitin, with the usual reservations about its purity; at least it affords a useful indication of the relative amounts of this important skeletal material.

The chromatograms were run on Whatman No. 1 paper using a mixture of *n*-butanol: pyridine: water in the proportions 30 : 20 : 13. This solvent is suitable for amino-acids as well as for sugars, but the amino-acids required a 40 hr run whereas the carbohydrates required 68 hr. The alcoholic solutions containing the haemolymph solids were spotted on to the paper so that comparable numbers of larvae were represented in each sample for analysis. Throughout this paper percentages are based on the dry weight of the insects.

The oxygen uptake of eighteen unfed hatchlings of *Locusta* and thirty-one of *Schistocerca* was measured individually in a Warburg respirometer at 30°. The insects were allowed to equilibrate in the apparatus for 30 min before readings were begun.

In certain additional experiments fed larvae or adults were also used. Hatchlings were sexed and weighed after the experiments in order to avoid handling the larvae prior to the estimation of their respiratory activity.

There are almost as many units of oxygen uptake in the literature concerned with locust physiology as there are authors. There can be little doubt that this lack of a common basis of comparison has obscured the inconsistencies of the earlier literature. In an attempt to provide such a basis, all the measurements quoted in Tables 4 and 5 have been converted into microlitres of oxygen per milligram total weight per hour.

The fat reserves of hatchling locusts

The earliest of our series of experiments were carried out using locusts fed entirely on grass, and the result of these individual analyses of hatchling Red locusts showed that many larvae emerge from the eggs of this species with virtually no reserves of fat at all, less than 50 μg per larva. Such larvae seem to survive

adverse conditions as well as those with more substantial fat reserves, and this fact alone discourages the view that fat is an important item in the metabolism of starving hatchlings. Our more recent series of experiments concern stocks of *Locusta* and *Schistocerca* fed on germinated cereals and bran and the artificial diet of Howden & Hunter-Jones (1958). Under these conditions, and with these species, considerable quantities of fat are transmitted from the mother to her offspring through the egg.

TABLE 1—INITIAL AND TERMINAL FAT CONTENTS OF HATCHLING LOCUSTS

Species	Number analysed	Phase	Fat content (%)	
			Initial	Terminal
Locusta migratoria	1942	*solitaria*	7·5	2·8
	1511	*gregaria*	10·7	2·2
Schistocerca gregaria	540	*solitaria*	5·3	2·4
	755	*gregaria*	9·3	4·9
Nomadacris septemfasciata	109	*gregaria*	13·0	2·8
Approximate standard errors:				
Locusta migratoria			±0·7	±0·3
Schistocerca gregaria			±1·2	±0·7
Nomadacris septemfasciata			±4·0	±0·5

Our analyses of aliquots of larvae drawn from individual egg-pods laid by these females show that the larvae from crowded females contain considerably more fat than do those derived from isolated parents. Table 1 gives the mean fat contents of freshly emerged larvae, and also the amounts remaining after the larvae have been starved to death. By comparing the initial and terminal fat contents of larvae issuing from the same egg-pods we can assess the amount of fat used up during starvation, even though the general level of fat varies from egg-pod to egg-pod so much that there are some lots of larvae which contained more fat after starvation than did others on eclosion.

According to Niemierko (1959) some of this residual fat may represent "bound" lipides not necessarily available for metabolism during starvation.

In one experiment on *Locusta* larvae individual analyses were performed on the issue from an egg-pod, one third of the larvae having been killed on eclosion and the remainder starved to death either at 35 per cent r.h. or at near saturation humidity. In neither instance was there any association between fat content and survival time; indeed, one of the larvae that most rapidly succumbed had a fat content even after starvation which exceeded that of all but one of the unstarved hatchlings.

If the fat contents of hatchlings are highly variable, they are not without a detectable relationship with the weight of the hatchling. Individual analyses of forty *Locusta* larvae, all from a single egg-pod, whose mean fat content was 4·0 per cent on a dryweight basis, disclosed a negative association between total weight and fat content such that the larvae had almost 1 per cent less fat for each 1 mg extra total weight. The actual regression coefficient is −0·95, significant at the 5 per cent level. The total weights of the larvae from this egg-pod ranged from

TABLE 2—CHEMICAL DIFFERENCES ASSOCIATED WITH COLOUR POLYMORPHISM IN THE *gregaria* PHASE OF *Schistocerca gregaria*

In each experiment only a sample of the black larvae issuing from the pod was analysed

Colour	No. analysed	Mean weight (mg)	Water content* (%)	Fat content† (%)
Larvae issuing from a pod laid in dry sand				
Black	28	14·25	73·01	3·62
Green	24	11·85	67·82	7·11
Larvae issuing from a pod laid in wet sand				
Black	34	21·80	81·94	2·54
Green	11	17·94	80·03	4·21

Approximate standard errors for weights ±0·14 mg
Approximate standard errors for water content ±0·27 per cent
Approximate standard errors for fat content ±0·60 per cent

*Based on total weight. †Based on dry weight.

8·8 to 13·8 mg. The range of fat contents, expressed as a dryweight percentage, was from 0·00 per cent to 14·14 per cent. A somewhat similar experiment was performed on the issue of a Desert locust egg-pod. Table 2 shows the differences of composition between the larger black hatchlings and the smaller green ones; the bigger larvae have the higher water content, relatively as well as absolutely (Blackith, 1961) and the lower fat content. In the case of the Red locust, *Nomadacris*, no absolute differences of fat content could be established.

Chromatographic analysis of the amino-acids extracted from hatchling locusts

The general pattern of the free amino-acid distribution in the first instar of all three species was remarkably similar. Indeed, no qualitative differences were observed, although some minor quantitative distinctions could be assessed. The *gregaria* of all three species contained glutamine, glycine, serine, threonine, alanine, proline, tyrosine and valine, all positively identified. In addition, all three contained two amino-acids which were probably leucine and *iso*-leucine, and also a substance staining blue with ninhydrin which is likely to have been histidine.

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Generally, the larvae from crowded parents contained higher quantities of amino-acids in the haemolymph than did those from isolated parents. For instance, threonine was notably augmented in *gregaria* hatchlings of *Locusta* as was the leucine–*iso*-leucine pair of spots. The glycine spot was more pronounced than that for serine in *solitaria* of *Locusta* but the reverse situation occurred in the corresponding *gregaria*. The supposed histidine spot was particularly strong in *Nomadacris* larvae, but that for tyrosine was weak in all three species. In *Schistocerca* the chromatogram was uniformly more intense for the *gregaria* than for the *solitaria*.

The suggestion has already been made that osmoregulation occurs in locust hatchlings by way of the lysis of haemolymph proteins. In order to explore this suggestion a comparison of the amino-acid spectrum of the blood of starved and unstarved larvae was included in these chromatograms. It is particularly interesting that those amino-acids which are differentially distributed between the phases are those which appear to be at least partially consumed during starvation. In particular, glycine and threonine seem to be used up in starved larvae, suggesting that some of the differential survival capacity of the larvae of the two phases may be associated with the composition of the haemolymph solutes.

Chromatographic analysis of the carbohydrates extracted from hatchling locusts

We have already reported that only small amounts of the simpler carbohydrates are present in the blood of hatchlings which have not had occasion to feed (Blackith & Albrecht, 1960). This situation contrasts strongly with the high concentrations of trehalose found in the blood of adults of the Migratory locust (Bücher & Klingenberg, 1958) and the Desert locust (Howden & Kilby, 1956). It is indeed remarkable that this ready source of energy should be so effectively excluded from the hatchling, since we have found that on starvation much of the trace of trehalose that is contained in the hatchlings can be used up. The glucose content of the hatchlings, on the other hand, is only slightly diminished on starvation. We have now established that small traces of both galactose and fructose occur in the hatchlings of all three species, and this seems to be the first authentic identification of free fructose in locusts.

The effective absence of glycogen from hatchlings has also been remarked upon (Blackith & Albrecht, 1960) and Howden & Kilby (1956) have shown that there is only a small amount of polysaccharide in locusts' haemolymph serum even in adults, amounting to less than 0·04 per cent of the weight of the blood, or perhaps 0·5 mg per adult insect.

There is, however, a prominent component of the hatchling haemolymph which can be found on a chromatogram suitable for the assessment of sugars. This may be the substance discussed by Dadd (1960) which he considers to be vitamin C. It is known that this substance is essential for the survival of locust hatchlings, and in particular that it builds up in the haemolymph prior to moulting, so that its exhaustion may entrain death at this critical stage of development. We have found that this substance is markedly enhanced in the offspring of crowded

parents, compared with its concentration in the blood of hatchlings from isolated parents of the same species, fed on the same diet as were the crowded parents. Moreover, this substance is almost entirely exhausted in *Locusta* hatchlings when these are starved to death so that again we are confronted with a substance, present in some quantity in the blood of hatchlings, which appears to be connected with the survival of the larvae in adverse conditions and which is also differentially distributed as between the phases.

The specific gravity of the locust haemolymph

The fact that adult locusts contain very much more sugar than do hatchlings, and the qualitatively narrow amino-acid spectrum of the latter, suggested that the specific gravity and probably the osmotic pressure of the hatchling haemolymph might be less than that of the adult. House & Stephens (1958) have made use of the gradient tube of Lowry & Hunter (1945) for the estimation of the specific gravity of the blood of *Melanoplus* spp. and we have also found this apparatus convenient and accurate. Small drops of the biological fluid are allowed slowly to sink into a thermally insulated tube in which a linear gradient of two liquids, miscible with one another but not with water, has been prepared. We found that dibutyl phthalate (s.g. 1·046) and tetralin (tetrahydronaphthalene, s.g. 0·971) are more suitable as the liquids of high and low density respectively, since the solubility of water in those specified by Lowry & Hunter is appreciable.

There is a clear cut distinction between the specific gravities of the blood of adult *Locusta* (1·0314) and that of the hatchlings (1·0257) when steps are taken to ensure freedom from regurgitated gut fluids. A similar difference was observed in the case of the Desert locust whose adults had a mean blood density of 1·0292 (males 1·0298; females 1·0285) whereas the first instar larvae contained blood of density 1·0220. These differences between the first instar and the adults are substantial by comparison with those between the sexes which, in common with House & Stephens, we found to be negligible. Other adult haemolymph specific gravities of acrididae are: *Nomadacris septemfasciata*, 1·0352; *Chorthippus parallelus* de G., 1·0306 (males 1·0293; females 1·0320); comparing with House & Stephens' data for *Melanoplus mexicanus*, 1·0261 and *M. bivittatus*, 1·0271. Howden & Kilby (1960) find that the total reducing value of the haemolymph is greatest in the fifth instar larva, when the specific gravity of *Schistocerca* haemolymph is 1·0397. Evidently, the instars which have fed on grass or cereals contain more sugars than do the unfed hatchlings. The sugar concentration is greatest in the late larval instars.

Further evidence that the fluid content of the hatchling locust does not contain its full complement of solids was obtained from the specific gravities of the gut fluid itself. In the case of *Locusta* hatchlings, the first instar larvae contain gut fluid of s.g. 1·0369, which is considerably less than the value relating to starved adults, whose gut fluid is approximately of s.g. 1·06. The gut fluids of *Schistocerca* were too strongly surface active for accurate measurement in the gradient tube.

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The chitin content of hatchling locusts

There are two outstanding results from our many analyses of chitin in locusts, especially in the first instar. Firstly there exist substantial phase differences, hatchlings from crowded parents containing less chitin than those from isolated parents. Secondly, even starved larvae are able to synthesize chitin, while they live, at a surprising rate, effectively doubling the chitin content, with which they emerge from the vermiform sheath, within a few days.

Aliquots of larvae from egg-pods laid by isolated Migratory locusts contained 5·74 per cent of chitin on a dry weight basis. In these experiments, some of the larvae, from each of the thirty-six egg-pods involved, were starved to death, and at that time, some 4 days after eclosion, they contained 10·14 per cent of chitin. Similarly, hatchlings from crowded parents contained significantly less chitin initially, 4·89 per cent, ending with 10·42 per cent. There were twenty-eight egg-pods from crowded parents, analysed separately.

In the case of the Desert locust, the differences were all in the same sense, but more strikingly different in the final rather than the initial chitin contents; ten egg-pods from isolated parents gave larvae having initially 5·17 per cent chitin, terminally 9·18 per cent, and thirteen pods from crowded parents gave larvae having initially 5·03 per cent and terminally 7·78 per cent of chitin. The same increase was found in the Red locust, in that thirty-seven insects from crowded parents (two egg-pods) moulted from the vermiform sheath with 5·07 per cent and died after starvation with 9·34 per cent of chitin.

Whatever the precursor of chitin in the hatchling, it must be present in quantity immediately after eclosion, since about half the total amount of chitin that is formed by the hatchling during the first instar is to be found by the end of the first 24 hr. For instance, a sample of larvae of gregarious *Nomadacris* from one egg-pod was divided into three lots of twenty larvae. One lot, analysed within an hour of eclosion, contained 5 per cent of chitin, a second lot analysed after 24 hr contained 8 per cent and after 5 days the larvae had a chitin content of 11 per cent. The development of this sample was deliberately retarded by keeping them at 20°C.

Similarly, some *Locusta* larvae which began with 6·7 per cent chitin, within an hour of eclosion, and had 7·6 per cent after 24 hr contained 8·0 per cent after 5 days at the same temperature.

Thus the hardening and darkening of the cuticle characteristic of the immediate post-eclosion period seem to be accompanied, in young locusts, by the formation of chitin itself.

Millot & Fontaine (1938) found some 5 per cent of chitin in the early instars of *Schistocerca*, and so did Rotman (1929) in hatchling *Locusta*, so that our results accord well with theirs as far as they go.

The secretion of chitin appears to be a continuous process throughout the life of the locust. The newly laid egg of the Desert locust contains virtually no chitin, whereas at the point of hatching it contains some 12 per cent of its dry weight, largely in the vermiform sheath whose shedding reduces the chitin content of the hatchling to the observed 5 per cent. In fact, some 81 per cent of the vermiform

sheath is insoluble in caustic potash solution. In the adult, too, there is an increase of chitin content on ageing. Pink, sexually immature, adult Desert locusts contain about 12 per cent of chitin, but this amount increases to some 16–20 per cent in senescent specimens.

Some effects of maternal age on the progeny of locusts

Under laboratory conditions, female Migratory locusts produce many egg-pods, and several series of the issue from these sequences of pods have been analysed for

TABLE 3—CONSTITUTION OF HATCHLINGS FROM A SEQUENCE OF EGG-PODS LAID BY A SINGLE CROWDED FEMALE

Pod No.	No. of Larvae	Total weight (mg)	Dry weight (mg)	Treatment Code	Fat (%)	Water-soluble solids (%)	Chitin (%)
	18			L	9·6	19·0	4·7
B.1	18	15·1	2·36	35	3·3	22·6	9·8
	18			100	1·9	23·4	7·0
	18			L	8·7	24·0	5·4
B.2	18	14·9	2·36	35	2·2	28·0	9·2
	18			100	0·8	29·3	11·2
	18			L	10·3	21·4	4·0
B.3	18	16·0	2·64	35	5·0	33·0	9·2
	18			100	1·8	24·7	10·7
	18			L	9·7	19·9	4·1
B.4	18	15·6	2·46	35	2·4	29·3	11·3
	18			100	1·9	28·6	10·5
	18			L	8·1	21·8	4·4
B.5	18	17·0	2·75	35	1·0	31·8	9·7
	18			100	1·2	29·5	9·3
	18			L	11·1	19·7	4·6
B.6	18	17·2	2·79	35	1·7	30·1	10·0
	18			100	2·3	27·5	10·3
	17			L	10·9	23·2	4·1
B.7	18	17·3	2·71	35	3·5	32·7	10·2
	18			100	2·4	28·7	10·6

Treatment code
L = killed and dried on eclosion.
35 = starved to death at 35 per cent. r.h.
100 = starved to death at 100 per cent. r.h.

wet and dry weight, fat content, water-soluble solids, and chitin content. These sequences all come from Dr. Albrecht's laboratory, and he has remarked on the consistent increase in weight of the progeny as the female ages. This increase is

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distributed evenly over the various constituents for which figures are available. Certainly the dry weight and wet weight run *pari passu*, as the data of Table 3 show. Two further series of seven pods from *gregaria* females have been analysed, as well as two series of fourteen pods each from *solitaria* females.

There is a suggestion that the earliest pods of each series are a little short of fat, but this tendency is not marked, and would not appear from the series shown. There is also a suggestion, in the long *solitaria* series, that the production of amino-acids as a response to starvation is more erratic in the first and last few pods of a sequence. In both sequences, the water-soluble solids are higher after starvation at 35 per cent r.h. than they are after starvation at saturation humidity, ten out of the fourteen pods having the difference in this sense in each case. There are no exceptions at all in the blocks of seven pods analysed after the first pair of each series. Since this trend can also be seen in the data of Table 3, relating to *gregaria* hatchlings, the suggestion that the production of extra water-soluble substances is a response to dehydration seems to be supported.

DISCUSSION

The unfed first instar of locusts retain some of the characteristics of the embryo. The incomplete differentiation of the gut fluid and haemolymph, for instance, and the need to maintain a high moisture content as compared with later stages; the low sugar content and narrower spectrum of amino-acids probably reflect the dependence of hatchlings on the maternal reproductive system rather than on external food supplies for body constituents. Wigglesworth (1943) notes that even large molecules such as those of haemoglobin may be transferred to insect eggs without necessarily having been synthesized in the reproductive system.

Such transfers constitute, in a sense, a mode of extranuclear inheritance. This inheritance may have trivial consequences, but may also play some part in the short-term regulation of growth and development.

At present we can influence the nature of this inheritance only by way of somewhat general maternal influences such as crowding and dietary changes. Moreover, in an exploratory investigation such as this, one can but hope that the analytical techniques, selected at least partly for expediency in processing large numbers of samples, will uncover differences between the phases, and between starved and unstarved hatchlings. In this way, the basic idea that survival may be related to the inherited reserves can be examined, since the differential survival of the phases is established. There is indeed an intimate connexion between those components differentially distributed between the phases and those exhausted on starvation, namely, threonine, glycine, the putative ascorbic acid and fat. The evidence for the value of high fat reserves in delaying death from starvation is, however, equivocal.

The fat reserves of hatchling locusts.

The role of the fat body of locusts as a site for biosynthesis may be at least as important as its role of an energy reserve (Kilby & Neville, 1957). This activity

is closely connected with the reproductive cycle in *Locusta* (Phipps, 1950) *Schistocerca* (D. Griffen, unpublished) and in *Melanoplus* (Weed-Pfeiffer, 1945). Slifer (1930) noted that only about half the fat transmitted by the mother to the egg of *Melanoplus* was used up in embryogenesis, but some preliminary experiments of ours on the developing eggs of *Locusta* suggest that 83 per cent is exhausted during the development of the egg of this species. Once the larva emerges from the egg its fat reserves are present not in the form of fat bodies, but dispersed in cells in the haemolymph, as Mathur & Soni (1937) found. The fat body in the usual sense of the term is not formed until well into the second instar (Coupland, 1957).

One of the most puzzling features of the distribution of fat in hatchlings is the large number of individuals which contain no detectable fat (less than 50 μg) even when they have been taken from a batch the average fat content of which is quite high. Laughlin (1956) points out that the coefficient of variation of the fat contents of the first instar chafers is high, between 38 and 48 per cent. Our coefficient for the spread of fat contents in hatchling locusts is not so extreme, being about 22 per cent, but even so, in a sample of *Locusta* larvae with a mean fat content of 6 per cent (fed on grass only) some 30 per cent of the individuals had no detectable fat; and in a sample whose diet included bran and hydroponic wheat seedlings the mean fat content was 9 per cent, but one in every ten larvae seemed devoid of fat. The biological interpretation of this distribution is complicated by the uncertainty as to whether the maternal ovarioles, or the developing embryos, are primarily implicated in this uneven allocation of fat to the hatchlings.

The spectrum of amino-acids in the haemolymph is consistent with that found by Treherne (1959) in adult *Schistocerca* except that his adults contained several amino-acids not found in our hatchlings. Duchâteau & Florkin (1958) found more glycine than serine in later larval instars of *Locusta* but noted that relative abundances fluctuate from stage to stage.

There is now substantial evidence that some insects, notably tsetse-flies, respond to starvation by consuming selectively a part of the amino-acid spectrum (Bursell, 1960) although the selectivity of tsetse-flies differs from that of hatchling locusts. Evidently, there is a tendency for selective consumption to counteract increases in free amino-acid content arising from proteolysis in the haemolymph.

No amino-acids appeared in the chromatogram of the blood of starved larvae that were not present in that of unstarved ones; it seems unlikely that the proteins which may be hydrolysed for osmoregulatory purposes contain component amino-acids not already present in the blood. There is probably an equilibrium in the lysis which can be shifted either way as a direct response to deprivation of food, and also to the disturbance of water-balance consequent upon starvation in these small insects.

Such phase differences as we have found are thrown all the more strongly in relief by the uniformity of the three species examined. One striking similarity is the synthesis of chitin, or at least of material insoluble in caustic potash solution, by the hatchlings of all three species.

This accretion of chitin represents an appreciable drain on the potential food reserves of the hatchling, since over 100 μg are produced in each hatchling of crowded stock and over 50 μg in each hatchling of isolated parentage. In the hope of identifying the precursors we have searched for glucosamine and N-acetylglucosamine in the haemolymph of the larvae, by chromatographic techniques, without success. Indeed, the total amount of sugars convertible into glucosamine is barely sufficient to account for the increased weight of chitin, and in fact we know that much of the sugar reserve is unchanged even after starvation to death.

There is a suggestion in the literature that glucosamine is itself toxic to insects. Rizki (1960) reports that about 5 per cent of glucosamine in a standard *Drosophila* medium phenocopied the lethal recessive gene "cryptocephal". We have sought to explore direct toxicity, rather than phenocopying, by submitting locusts to diets or injections containing glucosamine and also N-acetylglucosamine.

Diets containing 5 per cent and 10 per cent of glucosamine hydrochloride gave appreciably higher mortalities than occurred when N-acetylglucosamine hydrochloride at the same strength was fed to adult locusts, the acetylated derivative producing no higher mortality than control diets.

When 0·01 ml of a 10 per cent solution of either compound in insect Ringer was injected into the haemocoele of immature adult Desert locusts, 59 per cent of these insects injected with glucosamine hydrochloride were dead in 5 days, as compared with 23 per cent of the control insects injected with plain Ringer solution, and 41 per cent of those injected with N-acetylglucosamine hydrochloride. In these experiments each treated batch contained seventeen male and seventeen female locusts, the response being the same for the two sexes. These observations may be connected with those of Rosedale (1945) who could not obtain glucosamine as a product of the hydrolysis of the "chitin" of any stage of the Red locust after the first instar. Glucosamine has, however, been found in the tettigonid *Anabrus simplex* on acid hydrolysis of the cuticle (De Haas *et al.*, 1957). Although much remains to be done on this topic, glucosamine does seem to be toxic to locusts. The amounts of the obvious precursor, glucosamine, which were injected into adult locusts, were of the same order as the amounts likely to be present, if indeed glucosamine is the effective precursor for chitin synthesis. Glucosamine synthesised *in vivo* need not, of course, pass into the haemolymph, but the finding that such moderate doses are toxic even to adult locusts suggests that in this matter a little further work would be worth much more than speculation. If Rosedale's little-known findings concerning the insoluble fraction of the Red locust integuments are substantiated "chitin" synthesis by these insects may be different from the synthesis by other organisms.

The metabolic reserves of starving hatchlings

As a consequence of the large numbers of analyses performed on starving hatchling locusts we now have available an accurate estimate of the general metabolic activity of the insects after eclosion. In *Locusta*, for instance, we can demonstrate the effect of parental crowding on the utilization of food reserves by

hatchlings in a quite striking manner. The mean initial dry weight of larvae hatching from crowded stocks is 2·93 mg based on 500 insects, *solitaria* weighing 2·5 mg based on the dry weights of 650 insects. When starved to death, both phases lose weight, the *solitaria* losing 0·31 mg of which 0·12 mg is fat, the *gregaria* losing 0·48 mg of which 0·25 mg is fat. Thus both the absolute and relative amounts of fat metabolized by the larvae are the greater for the *gregaria* phase.

TABLE 4—OXYGEN UPTAKE BY HATCHLINGS OF *Schistocerca gregaria*

Mean O_2 uptake (μl/mg/hr)	Phase	Temperature (°C)	Reference
0·48	*gregaria*	29	Bodenheimer (1929)
0·90	*gregaria*	29	Gardiner (1958)
0·58	*solitaria*	29	Gardiner (1958)
1·65	*gregaria*	30	Blackith & Howden
1·71	*solitaria*	30	Blackith & Howden

Nevertheless, the greater part of the loss of weight on starvation is attributable to the metabolism of non-fatty materials, of which the *gregaria* lose 0·24 mg apiece and the *solitaria* 0·19 mg. These figures may be augmented to 0·34 mg and 0·24 mg respectively by the amounts of chitin synthesized during the period of starvation, amounting to 0·10 mg for *gregaria* and 0·05 mg for *solitaria*.

The problem of the identification of the major constituents of these non-fatty sources of energy can only be tackled at present by elimination of the likely candidates. We know from the chromatograms of the sugar contents of the larvae before and after starvation that the quantities of glucose and trehalose present cannot make a significant contribution, all the more so because the glucose content remains essentially unchanged after starvation. Our attempts to demonstrate glycogen in the hatchlings proved negative (Blackith & Albrecht, 1960). It is important to remember that only major body constituents are worthwhile candidates in this context, since we are searching for material to make up a deficit of about 14 per cent of the initial dry weight; as much, in fact, as the initial fat and chitin put together.

Blackith & Albrecht (1960) have already suggested that the osmoregulatory proteolysis may contribute to the energy reserve of starving hatchlings. Teppert (1958) considers that the amino-acids of *Melanoplus* eggs are partially converted to carbohydrates prior to combustion. It appears that amino-acids are important sources of energy in adult locusts (Clements, 1959) and in other insects (Bursell, 1960).

The variation of oxygen uptake from one individual locust to another is so great that only large differences between different groups of locusts differing in phase, sex, weight or other attributes could safely be discriminated from the sampling variation. We have been unable to demonstrate any systematic differences

of oxygen uptake according to the sex or weight of first instar larvae, nor do unfed hatchlings seem to respire differently from those that have fed on grass.

In addition to the high variability of individual locusts within any one stock, there are superimposed differences as from one stock to another. Evidently, any phase distinctions in terms of respiratory activity, such as have been claimed by Butler & Innes (1936) and by Gardiner (1958) are masked by this interstock

TABLE 5—OXYGEN UPTAKE BY *Locusta migratoria migratorioides*

Mean O_2 uptake (μl/mg/hr)			Phase	Temperature (°C)	Reference
Hatchling	Late larva	Adult			
0·75	0·24	0·34	*gregaria*	27	Butler & Innes (1936)
0·75	0·19	0·24	*solitaria*	27	Butler & Innes (1936)
—	0·77	0·48	*gregaria*	25	Krüger & Bishai (1957)
—	0·68	0·66	*solitaria*	25	Krüger & Bishai (1957)
Mean for individuals					
1·23	1·38	1·17	*gregaria*	30	Blackith & Howden
2·02	1·08	0·95	*solitaria*	30	Blackith & Howden
Mean for triplets					
0·94	—	—	*gregaria*	30	Blackith & Howden
1·20	—	—	*solitaria*	30	Blackith & Howden

variation (Tables 4 and 5). The one difference that appears consistently in our experiments is the substantial reduction of respiratory activity when three *Locusta* hatchlings were placed together in one respirometer bulb. The mean oxygen uptake for each member of these triplets is some 30 per cent less than that found for hatchlings tested individually (Table 5).

The fact that the respiratory quotient remains within the range 0·72–0·85 whether or not the larvae have fed suggests that the effective source of energy is not a carbohydrate. Although this conclusion is surprising in view of the ready source of sugars available in grass, it is quite consistent with the finding that sugars play only a small part in the metabolism of locust hatchlings.

We can now trace an association between the disappearance of glycine and threonine during starvation and the phase status, and hence the survival capacity, of the hatchlings, as well as the association with the disappearance of the ascorbic acid. From the chromatograms only a very rough estimate of the weights of these substances involved in metabolism can be made; the sum of the losses of these constituents cannot, however, be far short of the full deficit in question.

SUMMARY

(1) The first instars of the Red, Desert and Migratory locusts have been compared in respect of their initial contents of fat, amino-acids, sugars, a reducing substance which may be ascorbic acid, and chitin.

(2) The changes in these substances after starvation are assessed in relation to the survival capacity of the hatchlings.

(3) About half the loss of dry weight on starvation is due to fat metabolism, but individuals can be found without detectable fat reserves even in samples of hatchlings the average fat content of which is high; such individuals seem to survive as well as those rich in fat. Maternal diet influences hatchling fat contents.

(4) Trehalose is partly exhausted on starvation, although glucose remains unaffected. The sugars are present in much lower amounts than in the later instars.

(5) The putative ascorbic acid is present in the *gregaria* phase to a greater extent than in the *solitaria*, and this substance is also depleted during starvation.

(6) Although the blood amino-acid content of the *gregaria* phase is greater than that of the *solitaria* for most of the acids present, threonine and glycine in particular are exhausted on starvation. The three species differ little in amino-acid spectrum.

(7) Starving hatchlings double the chitin content in the few days that they survive unfed. Of the obvious precursors, glucosamine appears to be absent, and has indeed been shown to be toxic to locusts. No glycogen has been detected in the hatchlings. The source of the chitin remains conjectural.

(8) The variation of oxygen uptake from one stock of locusts to another is so great as to mask any differences attributable to phase or sex differences. Hatchlings fed on grass do not seem to have a higher oxygen uptake, or respiratory quotient, than unfed ones.

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MOVEMENTS OF POTASSIUM, SODIUM AND CHLORIDE IN INCUBATED GILLS FROM THE SILVER EEL

D. BELLAMY*

Department of Biochemistry, University of Oxford

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Abstract—(1) Isolated gills from silver eels adapted to tap water or sea water were incubated aerobically and anaerobically in either tap water or sea water. Net changes in the potassium, sodium and chloride content were determined.

(2) Under all incubation conditions isolated gills appeared to be almost completely impermeable to potassium.

(3) A constant level of sodium chloride was maintained in isolated gills under aerobic conditions against the normal diffusion gradient to which the eel was adapted. The constant sodium content appeared to result from a steady state in which passive diffusion was balanced by an active movement of sodium in the opposite direction.

INTRODUCTION

IN THE EARLY WORK on active chloride movement in the silver eel, intact gills were perfused with saline solutions (e.g. Keys, 1931; Bateman & Keys, 1932). The perfused gill technique suffers from the disadvantage that the effect of several variables on ionic regulation cannot be investigated in the gills of the same animal. This disadvantage can be avoided by using individual gills, isolated and treated by methods used to study ion movements in tissue slices (e.g. Koch, 1954). Thus, in the present work, changes in the ion content were determined when isolated gills were incubated aerobically and anaerobically in sea water or in tap water. The results obtained confirmed those of earlier workers on chloride movement, and, in addition, new observations were made on movements of sodium potassium and chloride in gills from silver eels adapted to fresh or sea water.

MATERIALS AND METHODS

Care of animals. Silver eels (*Anguilla anguilla* L.) were kept in metal framed glass tanks containing aerated tap water which was changed each week. The water temperature during the day ranged from about 6°C in winter to about 22°C in summer. Fish were adapted to salt water by transferring them for at least 48 hr to similar tanks containing filtered Plymouth sea water. Eels caught in fresh water have been termed "fresh water eels"; fresh water eels adapted to sea water have been termed "salt water eels". Oxford tap water (stored in glass) was found to contain 0·02 mM potassium, 0·44 mM sodium and 0·50 mM chloride. Plymouth sea water contained 12 mM potassium, 450 mM sodium and 510 mM chloride.

*Present address: Department of Zoology, University of Sheffield.

Dissection of gills. Eels were pithed and the gills exposed ventrally. After removing the strip of muscle covering the ventral aorta this blood vessel was ligatured behind the fourth gill arch. Individual gills were removed by cutting through each gill bar at two points, ventrally and dorsally where the gill filaments ended. As each gill was removed it was placed on cotton wool soaked in either tap water or sea water. Surface blood clots were removed from the gills by drawing them across wet cotton wool. From the bright red appearance of the gills a large amount of blood appeared to be retained in the gill filaments.

Incubation conditions. Isolated gills were incubated in 100 ml conical flasks containing 40 ml of either tap water or Plymouth sea water at $15 \pm 1°C$. Oxygen or nitrogen was continuously bubbled through the medium by means of polythene capillary tubes.

Extraction of gills. Gills were removed from the incubation media with forceps and rapidly dipped into about 500 ml of tap water. After draining off the surplus water by drawing them several times across a dry glass surface the gills were placed in test tubes and 2 ml of 0·1 N HNO_3 per gill was added. After 24 hr the nitric acid solution was decanted from the gills and used for the estimation of potassium, sodium and chloride.

Estimation of potassium and sodium. Potassium and sodium in nitric acid extracts of gills were estimated with the flame photometer described by Amoore *et al.* (1958), using LiCl as an internal standard.

Estimation of chloride. Chloride in 0·5–2·0 ml samples of the nitric acid extracts was titrated potentiometrically with 0·05 N $Ag(NO_3)_2$ using the method of Sanderson (1952) as modified by Bartley & Amoore (1958).

Estimation of iron. Portions of the incubation media (2·0 ml) taken before and after incubation of gills for 90 min were evaporated to dryness and the solids heated over a bunsen flame for 5 hr. The residue was dissolved in 3 ml 10 per cent HCl and the iron content of this solution was determined colorimetrically by the thiocyanate method of Varley (1958).

Radioactivity determination of Potassium. Radioactive $K_2{}^{42}CO_3$(1 mc in 0·5 g) was dissolved in 100 ml distilled water and 0·1 ml of this solution was added to 100 ml of sea water. Gills were incubated in 40 ml of the radioactive sea water, removed at intervals and extracted for 2 hr with 10 ml of 1·0 N HNO_3. The minimum radioactivity of the extracts measured in a liquid counter was 239 counts/min per 10 ml with a background of 15 counts/min per 10 ml.

Radioactivity determination of sodium. Radioactive $Na_2{}^{24}CO_3$ (0·25 g containing 1 mc neutralized with HCl) was made up to 100 ml with distilled water and 0·1 ml added to separate 100 ml portions of sea water and tap water. Gills were incubated and the incorporation of radioactivity into the gills was determined as for potassium above. The minimum radioactivity of the extracts was 142 counts/min per 10 ml.

Radioactive salts were obtained from the Atomic Energy Research Establishment, Harwell.

Wet weights. Gills were weighed on a 500 mg torsion balance after rapidly dipping in tap water and draining as described for the extraction of gills.

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Dry weights. Gills were weighed after drying in an oven at 110°C for about 18 hr.

RESULTS

Changes in the water content of incubated gills

The osmotic pressure of the blood in gills from fresh water eels was 30 times higher than that of the tap water in which the gills were incubated (e.g. Keys, 1933; Bellamy, unpublished). For gills from salt water eels the osmotic gradient was in the reverse direction, the osmotic pressure of the external medium being about 2·4 times higher than that of the blood. Therefore, if the gills were permeable to water, changes in the water content would be expected to occur in response to the osmotic gradients.

TABLE 1—CHANGES IN THE WATER CONTENT OF GILLS TAKEN FROM SILVER EELS AND INCUBATED IN TAP WATER AND SEA WATER

Four gills were dissected from one side of each fresh water or salt water silver eel as described in the text. The water content was determined after incubation for 1 hr (under the same incubation conditions as in Tables 2 and 3), and compared with the water content of unincubated gills from the other side of the animal. The figures refer to the average gain or loss of water per gill (from four determinations)

Gills from fresh water eels (incubation in tap water)		Gills from salt water eels (incubation in sea water)	
Eel No.	Change in water content (mg/hr/g dry wt.)	Eel No.	Change in water content (mg/hr/g dry wt.)
1	+235	6	−500
2	+320	7	−645
3	+260	8	−370
4	+375	9	−440
5	+395	10	−625

Water movements were measured by weighing before and after incubation. Gills from fresh water eels incubated in tap water showed an increase in water content, whilst those from salt water eels incubated in sea water showed a decrease in water content (Table 1). It is assumed that these changes were due to the osmotic influx and efflux of water. The average movement of water out of gills from salt water eels in sea water was about 60 per cent higher than that into gills from fresh water eels in tap water.

Changes in potassium, sodium and chloride of incubated gills

When isolated gills from eels adapted to fresh water and sea water were incubated in tap water and sea water respectively, the variations in potassium content of gills removed at different times of incubation were the same as those

between unincubated gills (Tables 2 and 3). Thus, there was apparently no change in the potassium content on incubation.

The sodium content of gills from fresh water eels in tap water fell initially between 10 and 20 per cent (Table 2). The drop in sodium was accompanied by the

TABLE 2—POTASSIUM, SODIUM AND CHLORIDE CONTENT OF GILLS FROM A FRESH WATER EEL INCUBATED IN TAP WATER

Gills were dissected from a fresh water silver eel and incubated in 40 ml tap water gassed with a continuous stream of oxygen at 15°C. Gills were removed at intervals and the ion content determined

Time of incubation (min)	Ion content (μM per g initial wet wt.)			$\frac{Na}{K}$
	Potassium	Sodium	Chloride	
0	40·1	62·0	43·9	1·55
15	42·0	51·3	32·5	1·22
30	42·7	52·4	32·5	1·23
45	42·3	48·9	32·0	1·16
60	38·4	50·3	33·4	1·31
90	41·5	50·5	32·1	1·22

loss of an equivalent amount of chloride. The incubation of gills from salt water eels in sea water produced a 20–30 per cent increase in sodium which was accompanied by an equal gain of chloride (Table 3). In each case the above

TABLE 3—POTASSIUM, SODIUM AND CHLORIDE CONTENT OF GILLS FROM A SALT WATER EEL INCUBATED IN SEA WATER

Gills were dissected from a salt water adapted silver eel as described in the text and incubated in 40 ml sea water gassed with a continuous stream of oxygen at 15°C. Gills were removed at intervals and the ion content determined as described in the text

Time of incubation (min)	Ion content (μM per g initial wet wt.)			$\frac{Na}{K}$
	Potassium	Sodium	Chloride	
0	47·2	81·5	76·6	1·73
15	46·5	111	107	2·39
30	48·0	115	108	2·40
45	44·8	110	109	2·46
60	47·5	110	110	2·32
90	46·7	108	106	2·31

changes were observed after 15 min incubation, but later work showed that most of the sodium chloride movement occurred in the first 5 min. Thereafter there were no further alterations in the sodium chloride content of gills from either fresh or salt water eels.

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It is likely that the initial increase in the sodium chloride of gills from salt water eels was due to a portion of the gill tissue equilibrating rapidly with the external medium. If this is assumed, it is possible to calculate the minimum size of this compartment of the gill in terms of tissue water. In the experiment given in Table 3 about 30 μM of sodium chloride per gram wet weight was taken up from the external medium with a sodium chloride concentration of 445 mM. If the sodium chloride gained was at the same concentration as that in the external medium the sodium chloride solution would occupy about 7 per cent of the tissue water.

It is possible to apply a similar calculation to the sodium chloride lost by gills from fresh water eels in tap water if it is assumed that a portion of the blood space of the gill comes into rapid equilibrium with the external medium. In this way it was calculated that the sodium chloride was lost from between 8 and 10 per cent of the tissue water.

From these calculations it appears likely that the initial sodium chloride movements in both media occurred in the same portion of the gills.

The part of the gill involved has not been identified. It is possible that the initial changes in sodium chloride content occurred by diffusion in the cut ends of the gill bar. The method of removing a gill from the eel meant that the terminal parts of the artery were unavoidably left open. Although the blood appeared to have clotted before incubation, and although there was no outward diffusion of haemoglobin (no increase in the iron content of the incubation medium) a net sodium movement could probably occur in the cut tissue. The initial movements of sodium chloride have been regarded as artifacts and all the rates of sodium chloride movement refer to the period after 10 min pre-incubation of the gills.

Exchange of potassium in isolated gills with potassium in the external medium

The results in Tables 2 and 3 show that there was no net change in the potassium content of isolated gills which were incubated in sea water or tap water. The same results were obtained when gills from fresh water eels were incubated in sea water, when gills from sea water eels were incubated in tap water and under all conditions when the stream of oxygen was replaced by nitrogen.

Although there was no net change in the potassium content a continuous exchange of potassium in the gills for potassium in the external medium was not ruled out. The latter possibility was tested by incubating gills from a fresh water and a salt water eel separately in sea water to which $K_2{}^{42}CO_3$ was added. Gills were removed at intervals and the specific activity of the gill potassium was compared with that in the sea water, which contained 3990 counts/min per μM K.

The specific activity of the potassium in fresh water gills increased during the first 45 min to 285 counts/min per μM K then remained constant for the rest of the incubation period (45 min).

Similarly, the specific activity of potassium in salt water gills reached an apparent equilibrium value at 145 counts/min per μM K. These results indicated that between 4 and 7 per cent of the gill potassium could exchange with that in the external medium. Because of the small fraction of potassium involved it is

possible that the exchange reaction was limited to potassium in the cut ends of the gill bars. It is likely that the gill filaments were impermeable to potassium.

Effect of incubation conditions on the ratio of sodium to potassium in isolated gills

Since the potassium content of isolated gills remained constant under the various experimental conditions it was possible to follow changes in the sodium content of incubated gills by measuring changes in the ratio of sodium to potassium.

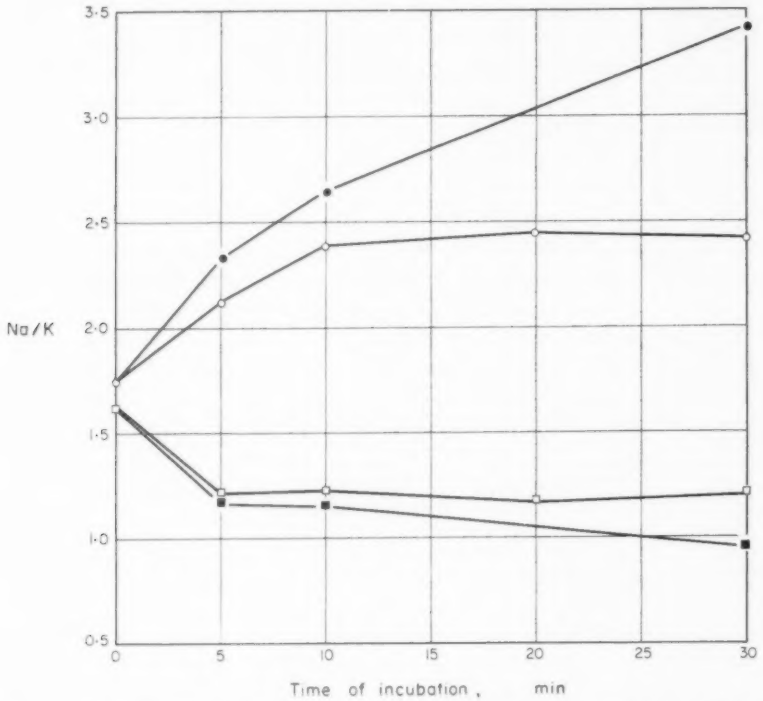

FIG. 1. Effect of incubation conditions on the sodium to potassium ratio of isolated gills. Gills from fresh water eels were incubated in tap water as described in the text either aerobically (□) or anaerobically (■). Gills from salt water eels were incubated in sea water aerobically (○) and anaerobically (●).

Fig. 1 shows that, aerobically, the ratio of sodium to potassium in salt water gills increased from 1·7 to 2·4 during the first 10 min incubation in sea water, then remained constant. The substitution of oxygen by nitrogen produced a greater initial rise in the ratio which then continued to rise throughout the whole period of incubation. In long term experiments in nitrogen, the ratio of sodium to potassium reached a maximum value after periods of between 45 and 90 min. The largest increase in the ratio which was obtained under anaerobic conditions was from 1·6 to 4·5 in 45 min.

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The ratio of sodium to potassium in fresh water gills fell from 1·6 to 1·2 during the first 5 min incubation in tap water, then remained constant (Fig. 1). In nitrogen the ratio continued to fall at a slow rate after 5 min and reached a value of 0·95 in 30 min and 0·65 in 60 min.

The average potassium content of gills from silver eels was about 15 μM per gill. In Figs. 2 and 3 the changes in the ratio of sodium to potassium which occurred

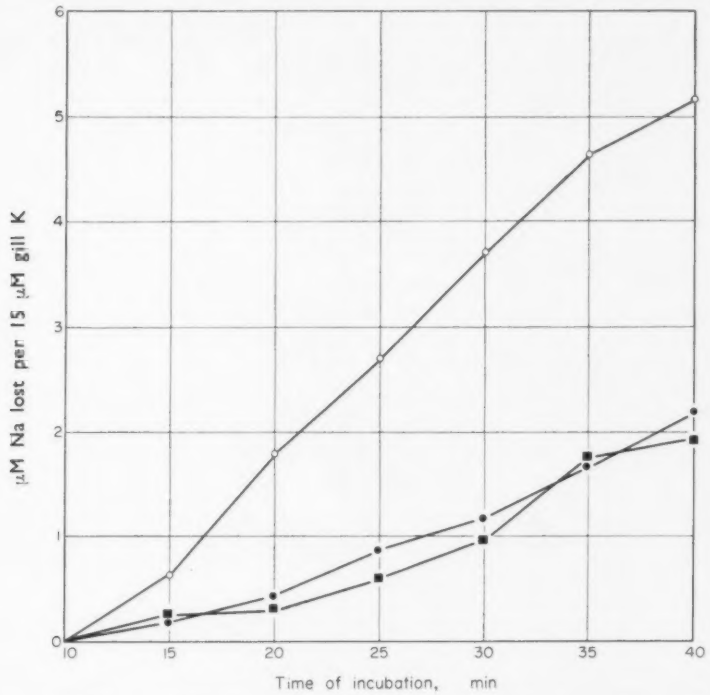

FIG. 2. Sodium lost from isolated gills incubated in tap water. Isolated gills were incubated aerobically and anaerobically in tap water after 10 min aerobic incubation in tap water. Gills from salt water eels in O_2 (○); in N_2 (●). Gills from fresh water eels in N_2 (■).

under the various conditions after 10 min incubation in fresh water or sea water have been expressed as a loss or gain of sodium per 15 μM of gill potassium.

In tap water (Fig. 2) the rate of sodium loss from anaerobic fresh water and salt water gills was the same. Under aerobic conditions the net sodium loss from fresh water gills was prevented but the loss from salt water gills was increased threefold.

In sea water sodium entered salt water gills (anaerobically) and fresh water gills (aerobically and anaerobically) at almost the same rate. Under conditions where sodium penetrated into gills in sea water, longer incubations (90 min) resulted in maximum ratios of sodium to potassium of between 3·2 and 4·5.

Rate of penetration of Na^{24} into isolated gills

When gills from fresh water and salt water eels were incubated in tap water and sea water respectively there was no net change in the sodium content after 10 min incubation. The constant sodium content under these conditions was dependent upon the presence of oxygen (Fig. 1). It was likely, therefore, that there was either a steady state balance of sodium between a passive loss and an active uptake in tap water, and a passive uptake and an active loss in sea water, or a sodium impermeability which was dependent upon the presence of oxygen.

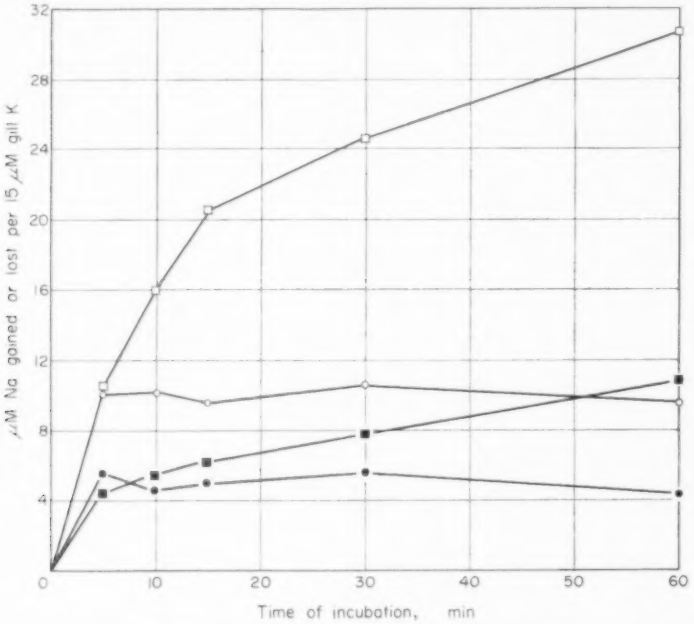

FIG. 3. Net changes in sodium content and the penetration of Na^{24} into isolated gills. Gills from a fresh water eel were incubated in tap water and the sodium content (●) and the penetration of Na^{24} (■) measured as described in the text. The sodium content (○) and the penetration of Na^{24} (□) were also measured for gills from a salt water eel incubated in sea water.

In order to distinguish between these two possibilities gills from fresh and salt water eels were incubated respectively in tap water and sea water to which $Na^{24}Cl$ was added. Gills were removed at intervals and the amount of sodium which had penetrated was calculated from the radioactivity in the gill extracts.

In Fig. 3 the rates of penetration of sodium, calculated from radioactivity measurements, are compared with the net changes in sodium content. For gills from salt water eels in sea water, although there was no net change in sodium content after 5 min incubation, radioactive sodium continued to penetrate the gills. After 60 min the specific activity of the gill sodium (72 per cent of that in the external medium) indicated that equilibrium had almost been reached. For gills

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from fresh water eels in tap water, after the usual initial drop in sodium content there was no further net change. However, from the continued increase in radioactivity after this time there was evidently an exchange of external sodium with that in the gills. This exchange occurred at a slow linear rate so that after 60 min the specific activity of the gill sodium had risen to 23 per cent of that in the external medium.

These two experiments indicate that aerobic sodium gradients are maintained although the gills are permeable to sodium. Further, the results suggest that the constant sodium content after 10 min incubation is due to a balance between a gain and a loss.

TABLE 4—RATES OF SODIUM MOVEMENT (μM SODIUM/15 μM GILL POTASSIUM/HR) IN ISOLATED INCUBATED GILLS FROM THE SILVER EEL

Net rates of sodium movement were measured after 10 min incubation. The figures (mean ± s.d.) are averages of several experiments with results similar to those shown in Figs. 2–4

Type of eel	Tap water		Sea water	
	O_2	N_2	O_2	N_2
Fresh water	+3·6* ± 2·2	−4·6 ± 2·7	+36·1 ± 6·3	+38·2 ± 8·4
Salt water	−7·0 ± 3·4	−4·1 ± 1·9	+35·6* ± 4·6	+39·2 ± 6·8

* No net change in gill sodium. Data from the rate of penetration of Na^{24} from the medium.

Rates of sodium movement in isolated gills

In all experiments where there was a net movement of sodium it was always found to be accompanied by an equivalent amount of chloride. A summary of the rates of sodium movement in isolated gills under different incubation conditions is given in Table 4. The rates are averages and refer to initial slopes (after 10 min incubation) obtained from several experiments similar to those shown in Figs. 1–3. It can be seen that in tap water the rate of sodium entry into gills from fresh water eels under aerobic conditions was the same as the rate of sodium loss from gills from both fresh and salt water eels in this medium. In sea water sodium entered gills from both fresh and salt water eels at almost the same rate under aerobic and anaerobic conditions. On the average the rate at which sodium entered isolated gills in sea water was about 10 times faster than the rate of sodium loss in tap water.

DISCUSSION

Comparison of the rates of water movement in isolated gills with those measured in the intact animal

Fresh water fish are said to gain water because the gill filaments are permeable to water and there is a large osmotic gradient for water movement into the animal

(e.g. Black, 1957). The excess water gained by this process is excreted as a urine which is very hypotonic to the blood. For eels in fresh water the average rate of urine production is about 2·5 ml per 100 g per 24 hr (Smith, 1929). When calculated on the same basis the average rate of water movement into isolated gills from fresh water eels in tap water was about 70 per cent of the above rate of urine production.

For the sea water eel the concentration gradient between tissues and environment is in favour of a passive water movement out of the body. To counterbalance this osmotic water loss the eel excretes very little urine (Smith, 1929) and absorbs water from the gut. The average water loss by isolated gills of the salt water eel incubated in sea water is almost the same as the rate of water absorption from the gut of salt water eels as measured by Smith (1930) and Keys (1933). These comparisons show that isolated eel gills behave osmotically as predicted from physiological experiments on the whole animal.

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Sodium balance in the gills of salt water eels

When isolated gills from salt water eels were suspended in sea water the constant sodium content appeared to be due to a steady gain of sodium which was balanced by an equivalent loss. The sodium concentration in sea water was more than twice as high as that in eel serum. Thus a passive diffusion of sodium into the gills would be expected if the external membrane was permeable to sodium. Sodium entered salt water gills at the same rate both aerobically and anaerobically but under the latter condition there was a net gain of sodium. This indicates that there is normally a steady state level of sodium in the gills of salt water eels suspended in sea water which is due to a passive entry of sodium balanced by an active output.

It was shown by Keys (1933) that there was no net movement of chloride out of gills of the salt water eel perfused in sea water until the chloride concentration in the perfusate was higher than that in normal eel serum. Thus, the present work fits in with the idea that in the salt water eel, sodium is constantly being removed from the gills by an active process to balance an inward flow by diffusion, and that the output mechanism can adjust itself to excrete additional sodium which is absorbed along with water from the gut.

Sodium balance in the fresh water eel

The fresh water eel in tap water excretes urine containing between 39 and 116 mM sodium chloride (Sharratt, unpublished). By analogy with processes which are known to occur in other fresh water fish (Krogh, 1939) it is likely that the loss of sodium chloride by the eel is made good by an active uptake from the external environment by the gills. The uptake of sodium by the eel in fresh water has not been previously demonstrated but the present results indicate that there is a constant active movement of sodium into the gills to balance a loss by diffusion. It is likely that in the intact fresh water eel the active uptake process could serve to replace any sodium chloride lost in the urine.

The results show that the rate of sodium diffusion out of the gills in tap water is the same for both fresh and salt water eels. Similarly there is no difference between the rate of sodium diffusion into gills from fresh and salt water eels in sea water. Although there is a larger sodium concentration gradient in tap water the diffusion rate is 10 times greater in sea water than in tap water. If sodium diffuses in and out of the gills through the same site, free diffusion outwards appears to be greatly impeded. Alternatively two sites may allow a one way diffusion of sodium at different rates.

The sites for sodium diffusion in the eel may reflect its euryhaline nature which allows an apparent reversal in the direction of active sodium movement in the gills. For instance, two sites for the active transport of sodium may occur in the gills which permit the passive diffusion of sodium. Sodium diffusion would only take place when the direction of the sodium concentration gradient required to activate a given transport mechanism was reversed.

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SHORT COMMUNICATION

THE EFFECT OF TEMPERATURE CHANGES ON THE SPONTANEOUS NERVOUS ACTIVITY OF THE ISOLATED NERVE CORD OF *LUMBRICUS TERRESTRIS*

M. S. LAVERACK*

The Nature Conservancy, Merlewood Research Station, Grange-over-Sands, Lancashire

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Abstract—1. The effect of changing temperature upon the spontaneous nervous activity of the central nervous system of *Lumbricus terrestris* has been investigated.

2. The curves obtained for steady rate discharges at various temperatures show optima in the range 13–17°C.

3. Nervous responses are of three types (a) no alteration in frequency over a wide range of temperatures, (b) a speeding up on warming and a slowing down on cooling, and (c) an anomalous transient in which warming causes an initial slowing of the rate, and cooling causes an initial increase in the rate.

4. The earthworm thus shows responses to temperature similar to those of the slug, crayfish and cockroach.

EARTHWORMS can be expelled from soil by the application of dilute formaldehyde to the surface of the ground, and this has been used (Raw 1960) as a means of sampling populations of *Lumbricus terrestris*. In a population of this species studied by Satchell (personal communication) the proportion of the population coming to the surface following formaldehyde treatment was found to be related to soil moisture and temperature conditions. It was positively correlated with soil temperature in the range 0–12°C, but at higher temperatures lower numbers of worms were recovered. This may reflect a seasonal fall in the population density or a reduced response to the formaldehyde stimulus and work is in hand to determine the influence of each of these factors. The present paper describes experiments concerned with elucidating the effect of temperature on the intrinsic nervous activity of the isolated nerve cord of *L. terrestris* and which may affect the activity of these animals in the field.

The method used closely follows the scheme of Kerkut & Taylor (1958). The central nerve cord of *L. terrestris* was removed from an unanæsthetized animal and placed in a drop of Frog Ringer solution on the stainless steel lid of a perspex box. Water of various temperatures was passed through the box and the temperature recorded by a thermistor lying directly under the lid. The lid was connected to

* Present address: The Gatty Marine Laboratory, St. Andrews, Fife.

earth and nervous potentials recorded via a glass insulated tungsten electrode inserted into the nerve cord. The display was via an amplifier and oscilloscope. Magnetic tape recording was used to facilitate analysis at a later stage. In most cases potentials from only a few units were obtained. The frequency of nervous impulses was measured by a dekatron scaler (modified from Kerkut, 1955) and in the case of multiple unit activity a valve diode system was used to discriminate between larger and smaller potentials.

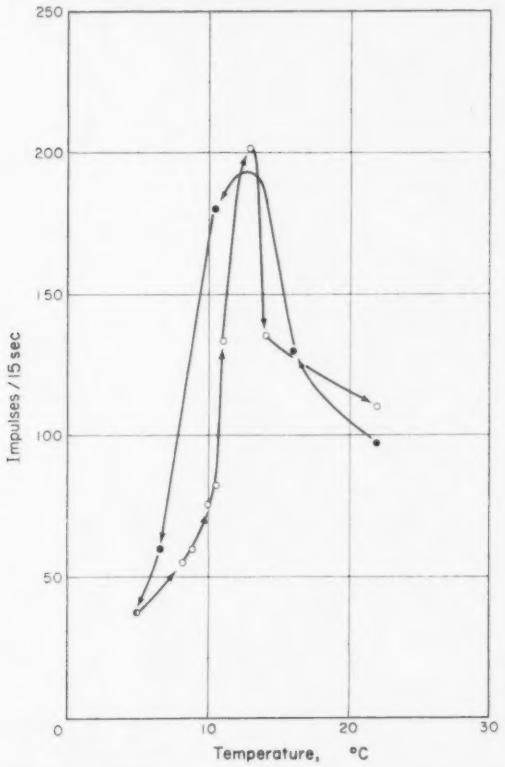

FIG. 1. Graph showing steady rate curve of nervous activity for a range of temperatures. The number of impulses rises towards an optimum at 13°C and then falls as the temperature increases further.

The spontaneous nervous activity within the nerve cord was first measured at room temperature (18–20°C). The temperature was then changed, both above and below this temperature, the preparation allowed to remain at the new temperature for 3 min and further measurements made. The new frequency was characteristic for each temperature and remained steady for as long as the temperature remained unaltered.

In Fig. 1 the results obtained from one such experiment are plotted. The frequency was low at 5°C, and mounted to a maximum at about 13°C, falling again above this temperature. Although the actual shape of the curves differs somewhat according to whether the temperature is raised or lowered, the optimum occurs at

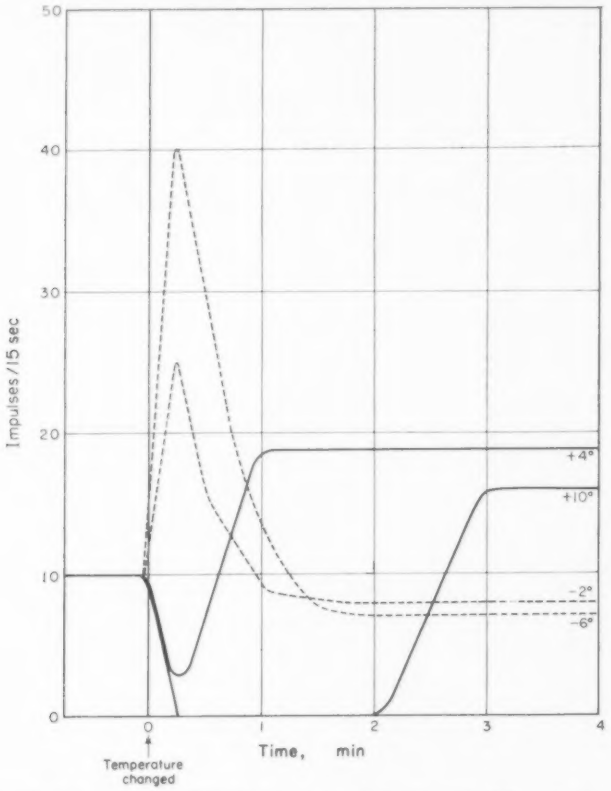

FIG. 2. Graph to show the effect of temperature changes of different magnitudes. It indicates that an increase in temperature causes a transient decrease in frequency and a final rate higher than the initial. The duration of the transient is much larger and more pronounced for a change of 10°C than for 4°C. A decrease in temperature is followed by an increase in frequency which quickly falls to the new level. The final rates depend on the temperature relative to the optimum as explained in the text.

the same temperature throughout. The optimum temperature varied from animal to animal and in seven experiments it occurred at 14–16°C in four, at 13°C in one and at 16-17°C and 17–18°C in one each This range may reflect the previous history of the animal in the field and indicate acclimatization.

The alteration of the temperature of the nerve cord influenced the impulse frequency in three ways. Firstly, there may be no response, secondly some cells

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may act as heat receptors and increase the rate of discharge as the temperature rises, or thirdly the cells may show anomalous transient responses.

The first of these responses is the least common. Some rhythmically firing cells show a wide tolerance of temperature, the frequency remaining virtually unaffected over the range 5–30°C. It is, however, very noticeable that as the temperature declines so also does the size of the potential. Recovery to the original size occurs again when the temperature rises. In general these cells and all others as well become intermittent and eventually cease to discharge at temperatures above 30°C.

The second type of response is the normally expected type. An increase in temperature is followed by an increase in activity, and a lowered temperature leads to a decrease in frequency. In a typical experiment a decrease from 7·4 to 5·4°C had the effect of slowly decreasing the activity of a single nerve cell from 3/sec to 1/sec. When the temperature was raised from 16·6 to 33°C the activity increased from a slow rate to a series of high frequency bursts that continued for a considerable period. It can well be assumed that these bursts would give rise to rapid avoiding movements of the whole animal as reported by Smith (1902) for *Eisenia foetida* at this temperature. When the temperature is again lowered the cells return to a steady slower rate. The frequency of these cells is sensitive to a temperature change of only 1°C.

Most commonly the nervous activity demonstrates anomalous transients when the temperature changes. A decrease in temperature leads to a burst of activity, the size and duration of which depend upon the magnitude of the temperature change (Fig. 2), but the final rate is lower than the initial rate if the temperature was below the optimum at the start. A contrary response is noted when the temperature is raised; the nervous activity shows a decrease in frequency which may include a complete cessation of activity lasting up to 2·5 min when the temperature change is considerable (Fig. 2). The final rate at which the preparation is active depends upon the temperature relative to the optimum temperature. If the temperature is raised from the optimum then the final rate is lower than the initial rate; if the temperature is dropped from above towards the optimum then the final rate is higher than the initial rate. Opposite effects are noted when the temperature of the preparation is originally below the optimum.

The occurrence of anomalous transient responses of nervous activity after stimulation by changing temperature has previously been reported in other invertebrates such as the slug, cockroach and crayfish (Kerkut & Taylor, 1958). To this list can now be added the earthworm. The time course and correlation of size of response (transient frequency) with the magnitude of temperature change is very similar to those of the slug and cockroach, taking up to 2·5 min to reach completion and a new steady rate.

The shape of the steady rate curve suggests that the earthworm *L. terrestris* may be most active in the field when the soil temperatures range between 13 and 18°C and that beyond these limits activity falls. This may partly explain the drop in numbers obtained by chemical sampling during the winter and summer as compared with spring and autumn.

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BOOK REVIEW

G. A. KERKUT: **Implications of Evolution.** Vol. 4 in Zoology Division, International Series of Monographs on Pure and Applied Biology. Pergamon Press, Oxford, 1960. x+174 pp., 30*s*., $5.

THE AUTHOR of *Implications of Evolution* has made a thoughful attack upon the tendency of teachers and students of zoology to regard as facts a number of evolutionary concepts that are better regarded as unproved assumptions. In this respect the book has a worthwhile objective and is refreshing. However, it may be said in criticism that the author often adopts a negativistic approach, and his way of going about his task seems at times to be based on the method of setting up straw men, which he then proceeds to demolish. Thus, in Chapter 2, Dr. Kerkut lays out seven "Basic assumptions"; these are:

1. That non-living things gave rise to living material.
2. That this event occured only once.
3. That viruses, bacteria, plants, and animals are all interrelated.
4. That the protozoa gave rise to the metazoa.
5. That the various invertebrate phyla are interrelated.
6. That the invertebrates gave rise to the vertebrates.
7. That the relationships between vertebrate classes are understood (what the author says here is that "the fish gave rise to the amphibia, the amphibia to the reptiles . . . " etc., but from the later text it is not quite clear what is meant).

The author then goes on to state (p. 7), "I shall consider that the supporters of the theory of Evolution hold that all these seven assumptions are valid, and that these assumptions form the "General Theory of Evolution." In short, Dr. Kerkut has defined a general theory in terms so rigid as to make it a veritable Maginot Line of evolution. It is difficult to imagine that most modern zoologists would attempt to defend every part of it; the majority would take to the hills, leaving Dr. Kerkut to press his attack. Perhaps the strongest defence could be made of the first assumption, but we find that Dr. Kerkut's objections are not directed to the stated assumption at all; rather his contention is that we do not yet know how life arose, and that we have as yet no proof that one or other theory of the origin of life is correct. The assumption that living substance arose from the non-living can be made or not as we wish; we can study the evolution of life without any assumptions as to its origin, but Dr. Kerkut's criticisms seem not to make the assumption either less or more necessary. The matter may not be capable of being proved; we operate in the realm of inference and relative plausibility.

The second assumption is actually so widely questioned at the present time that it seems hardly necessary to insist on its being basic to a "general theory" of evolution. In support of Kerkut's thesis that the origin of life has not been proved to have been a unique event, the reviewer notes that if an event might have occurred any number of times, the probability of its having occurred just once is less than that of its having occurred some unspecified number of times, and it is not a valid application of the principle of parsimony to assume as the simplest explanation that life arose at a single point in space and time.

Assumptions 3–7 are said by the author to follow from the first two, although the reviewer fails to see the necessity of this. Point 3 is attacked on the grounds that we still cannot determine the form of the relationship between viruses, bacteria, rickettsiae, protozoa, protophyta, and multicellular organsims. Assumption 4, that the Protozoa gave rise to Metazoa, is attacked on comparable grounds, namely, that we really don't know just how Metazoa arose, and whether or not multicellularity in animals arose more than once. The vehemence of arguments on this matter among zoologists in recent years should make it evident that the "assumption" posed by Kerkut is certainly not one on which zoologists as a group are in agreement.

Assumption 5, "that the various invertebrate phyla are interrelated", is likewise attacked on the grounds that there are current differences of opinion on how these groups are interrelated. It does not seem to this reviewer that Dr. Kerkut succeeds in demolishing the assumption as stated, and the same could be said for his treatment of assumption 7. Great areas of doubt and disagreement are exposed, but the assumptions are defeated only when presented in a rigid form. Chapter 6, "The Most Primitive Metazoa" and chapter 7, "The Invertebrate Phyla", do not impress the reviewer as representing especially good zoology; there are a number of weak generalizations, errors, and omissions that suggest inexperience on the part of the author. However, chapter 8, "Biochemical Studies of Phylogeny", includes a very critical and informative discussion of the phylogenetic evidence provided by the phosphagens: arginine and creatine phosphates and certain comparable compounds. If this discussion alone could reach into the hundreds of elementary courses where it is now being presented in incomplete, oversimplified, and out-of-date form, the book would justify its existence.

Assumption 6 is not discussed; the reader is referred to Berrill's *Origin of the Vertebrates* for an example of how one's views on this matter must depend on certain assumptions, and it may be agreed that, as in a novel, the quality of the work should be judged by how well the author develops his stated assumptions.

In his conclusion, Dr. Kerkut reiterates that what he has been discussing is (p. 157) "the theory that all the living forms in the world have arisen from a single source which itself came from an inorganic form. This theory can be called the 'General Theory of Evolution'". This reviewer feels that the treatment of this thesis has been a bit like kicking a man when he is down. However, this may not always be without salutary effects in the long run. Zoology, like biology in general, has arrived at a state where the accumulated factual data reveal a complexity that can no longer be unified by the simple schemes of the past. In particular, we need to impress on the student the need to be aware of the gaps in knowledge so that his future researches may better illuminate them. We should urge that instead of having elementary students learn "a phylogeny" which they accept as a fact or a truth (as judged by a good examination score), each student should attempt to construct the particular phylogenies which could be derived from a given set of clearly stated assumptions when these are viewed in relation to what may be regarded as "facts". We have found this a valid approach in teaching, and my guess would be that the hapless undergraduate who serves as Dr. Kerkut's "straight man" in Chapter 1 would emerge from his ordeal a more thoughtful and less complacent young biologist.

RALPH I. SMITH

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THE EFFECTS OF DRUGS ON THE NEURONES OF THE SNAIL *HELIX ASPERSA*

G. A. KERKUT and R. J. WALKER

Department of Physiology and Biochemistry, The University of Southampton

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Abstract—(1) The effect of adding various chemicals to the isolated brain of the snail *Helix aspersa* is described. The reactions were followed by measuring the effect of the chemicals on the resting potential and the spontaneous action potentials of the nerve cells.

(2) All the following chemicals were effective at dilutions between 10^{-7} and 10^{-11} g/ml. The sign indicates their overall acceleratory (+) or inhibitory (−) effect on the spontaneous activity.

Acetylcholine (+)	Glutamic acid (±)
Adrenaline (+)	Histamine (±)
Cocarboxylase (±)	Nor-adrenaline (±)
Dimethyl-amino-ethanol (−)	5-HT (+)
Dopamine (−)	Phenylalanine (−)
γ-Aminobutyric acid (+)	Thiamine hydrochloride (±)

(3) Dopamine and phenylalanine caused a hyperpolarization of the resting potential.

(4) Phenylalanine, glutamic acid and cocarboxylase could protect the cell against the action of high concentrations of acetylcholine.

(5) It is thought that most of the above chemicals may occur naturally in the snail and that acetylcholine and dopamine are the most likely transmitter substances.

(6) Most of the drugs had a dual action; inhibiting some cells and accelerating others. It is suggested that there is a marked chemical heterogeneity in the CNS with cells reacting specifically but differently to a given chemical.

INTRODUCTION

IN RECENT YEARS interest has been aroused in the study of the pharmacology of the invertebrates. Various invertebrate preparations such as the leech muscle (Minz, 1932; Feldberg & Krayer, 1933) and the heart of *Venus mercenaria* (Welsh & Taub, 1948) have proved of especial use in drug assay. The actions of various drugs have been determined for selected invertebrate preparations: i.e. anemone sphincter muscle (Ross, 1960a, b); cockroach nerve cord (Twarog & Roeder, 1957); crayfish nerve cord (Wiersma & Schallek, 1948; Hichar, 1960a). The molluscan nervous system has been to some extent neglected and the present paper will describe a series of experiments on the effect of various naturally occurring substances on the resting potential and the spontaneous activity of neurones from the common garden snail *Helix aspersa*.

The compounds used were either naturally occurring substances or precursors of such substances, and in the main they were chosen so as to allow some insight into the nature and action of the chemical transmitters at the synaptic junction.

METHODS

The experiments were carried out on brains from locally collected snails *Helix aspersa*. The brains were dissected and placed on a glass slide. The connective tissue surrounding the brain was carefully removed and the brain then placed on a black glass slide and then put in a small (10 ml) bath containing Cardot's ringer (1921). 5–30 megohm glass microelectrodes filled with 3M KCl were inserted into the nerve cells of the parietal and visceral ganglia. The electrodes led to a cathode follower and thence to a Tektronix 502 oscilloscope. The potentials were monitored on a twin channel tape recorder and simultaneously led to an oscilloscope camera. The tape records were replayed either onto an Ediswan Pen recorder or the camera.

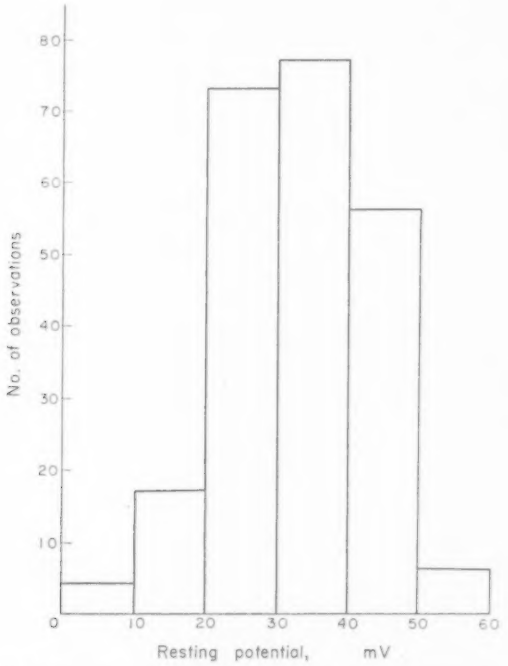

FIG. 1. The resting potential in snail nerve cells. The frequency of obtaining the various potentials is plotted. The majority of the neurones had a potential between 20 and 40 mV.

The test solutions were usually made up and stored in a refrigerator. They were diluted to the required range just prior to the experiment. All concentrations were added in series, the most dilute being used first of all. The final volume of the bath led to a tenfold dilution of the added material.

All the drug concentrations are expressed in terms of grams per millilitre. Thus 10^{-6} means that 1 ml of the final solution would contain one millionth of a

gram of material. The only liquid used, DMAE, was made up v/v instead of w/v. Dilutions greater than 10^{-6} are considered as being physiological whilst those less than 10^{-6} are considered as being pharmacological levels.

Many of the winter snails that were used were inactive and in a hibernating condition. It had previously been found that such animals had nerve cells with a low resting potential and very little spontaneous activity (Kerkut & Walker, 1961). The animals were brought into activity in the following manner. The snails were placed in a jar containing $\frac{1}{4}$ in. of water over crumpled chromatography paper. The lid of the jar was screwed down and the jar then brightly illuminated with two bench lamps for a couple of hours. The results of the humid atmosphere, the light and possibly the increased temperature led the animals to emerge from their shells and crawl round and round the jar. The brains of such animals were indistinguishable from those of normally active animals; they had a high resting potential and many of the cells were spontaneously active. During the summer and autumn, normal active snails were used from the garden. No difference was noted between the reactions of activated snails and normally active snails.

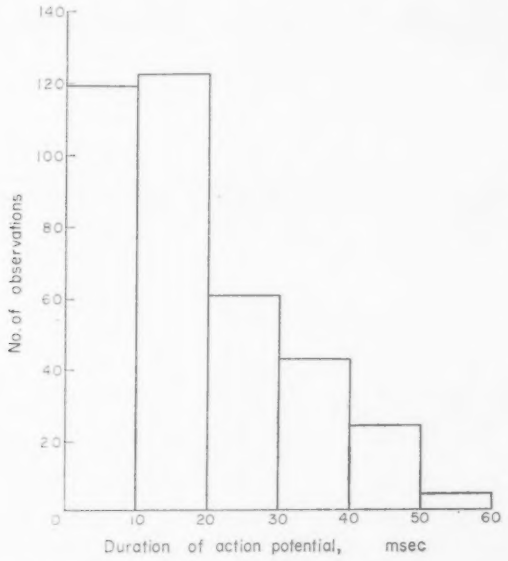

FIG. 2. The duration of the action potential. The frequency of obtaining action potential of specified duration is presented in the above histogram. The majority of potentials were less than 20 msec duration, but some were as long as 60 msec.

RESULTS

(a) *The properties of the resting potential and the action potential*

The resting potential varied in value between 7 and 50 mV. Fig. 1 shows a histogram of the frequency distribution of 247 resting potentials all of which were

taken from nerves showing spontaneous activity. Of these values, 210 fell between 20 and 45 mV, with the maximum number of cells having a potential between 30 and 39 mV. The degree of depolarization that occurred preceding each action potential varied slightly from one neurone to the next, but in forty-eight out of sixty-six cells where this phenomenon was carefully noted, the depolarization was between 4 and 6 mV; i.e. the resting potential fell this amount before the action potential was initiated. The complete range itself lay between 2 and 22 mV.

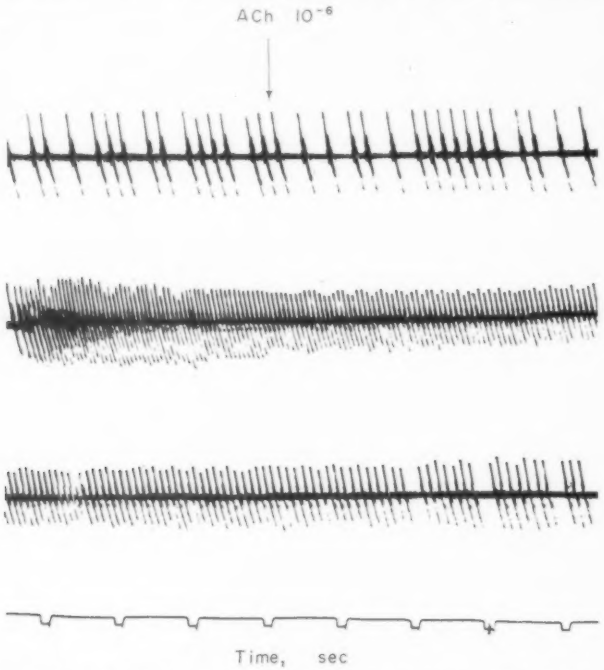

FIG. 3. The action of acetylcholine. This figure is a pen recording of the activity of a single neurone, the three parts of the figure being continuous. Acetylcholine at 10^{-6} g/ml was added at the arrow, and the nerve responded 4½ sec later by acceleration.

The action potential varied slightly in shape from one neurone to another, though "typical" action potentials with overshoots of 20–30 mV were often found. On other occasions, however, the action potential would fail to overshoot the base line, and the action potential would thus be less than the resting potential. The pattern of potential was constant for any one neurone under observation. On occasions multiple spikes could be observed, the spikes firing off at independent frequencies; on other occasions the post synaptic potentials could be clearly seen. Yet other occasions exhibited a double synchronous spike. The duration of the action potential showed a considerable range, lasting from 2 to 100 msec. Fig. 2 shows the frequency distribution of the duration of the action potential in 374 different cells. The majority of the potentials were of short duration.

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(b) *The effect of drugs on the spontaneous activity of snail neurones*

1. *Acetylcholine (ACh).* Acetylcholine at physiological levels was found to affect the spontaneous activity of nerve cells. Fig. 3 shows a pen recording of an experiment in which the preparation responded to the addition of 10^{-6} g/ml ACh. There was a delay of approximately 4 sec and the activity of the unit then markedly increased. This increased activity was maintained for over 20 sec. The most sensitive preparations responded to 10^{-8} g/ml, the response being an increase in the spontaneous activity.

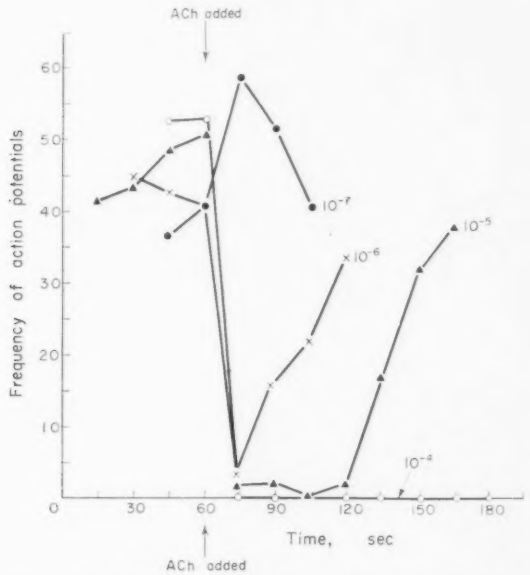

FIG. 4. The action of acetylcholine. The effects of adding various concentrations of acetylcholine to a single preparation are shown in this graph. 10^{-7} g/ml caused an acceleration; 10^{-6} caused a slight inhibition. The inhibition was more marked at the concentrations 10^{-5}, 10^{-4}.

Fig. 4 shows the effect of different concentrations of ACh on the activity of a single nerve cell. 10^{-7} caused a slight increase in the activity; 10^{-6} caused a decrease in activity that lasted for 1 min, after which the activity increased. Higher concentrations of ACh caused an increase in the duration of the inhibition. This experiment demonstrates that ACh has a dual effect on the spontaneous activity; it can cause an increase in activity and it can also cause a decrease in activity—the difference sometimes depending on the concentration of the ACh. Other cells have been observed which would only show inhibition following acetylcholine addition. The maximum dilution that has been observed to bring about inhibition is 10^{-7} g/ml. Higher concentrations of ACh (10^{-4}) caused a

brief increase in activity and then a marked inhibition. This was usually accompanied by a lasting depolarization of the resting potential almost to zero. Levels such as 10^{-5}, 10^{-6}, 10^{-7} rarely had such a marked effect on the resting potential.

Certain chemicals tended to protect the membrane against the action of 10^{-4} ACh; they either prevented the complete depolarization or tended to make the depolarization last for a shorter time. This effect was most marked after application of β-phenylalanine > glutamic acid > cocarboxylase, the drugs being arranged in order of decreasing protection against ACh. γ-Aminobutyric acid and noradrenaline did not appear to provide any such protection to the membrane.

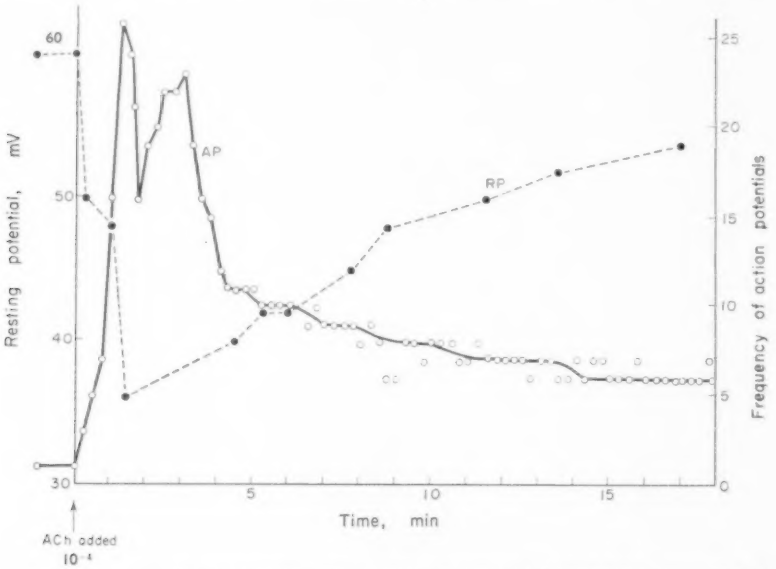

FIG. 5. The action of acetylcholine. The resting potential (●) and the frequency of the action potentials (○) are shown in the figure. Acetylcholine caused a sudden decrease in the resting potential and an increase in the frequency of the action potentials. As the resting potential returned towards the initial level, the frequency of action potential decreased.

Fig. 5 illustrates the relationship between the resting potential and the frequency of the action potentials. In this particular case the membrane had been pretreated with phenylalanine. ACh at a concentration of 10^{-4} was then added. It will be seen that the resting potential fell from 60 to 36 mV whilst the action potentials increased in frequency from 1 per 15 sec to 30 per 15 sec. As the resting potential slowly recovered, so the frequency of the spikes decreased. Had the nerve been treated to 10^{-4} without any pre-treatment to phenylalanine, the resting potential would have fallen to zero and remained there. The action potentials would, after an initial increase in activity, have been completely inhibited.

This experiment is also of interest in that it indicates that the drugs are acting on the actual cell under observation. The electrode would measure the resting

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potential only of the impaled cell and the activity of this cell closely followed the resting potential values. This fact is of importance in considering the action of drugs on the cells in the brain since the cells remain in contact with one another and it is often difficult to decide whether the effect that one sees is a direct effect of the drug on that cell or whether the drug has acted on other cells that are in synaptic connexion with the observed cell.

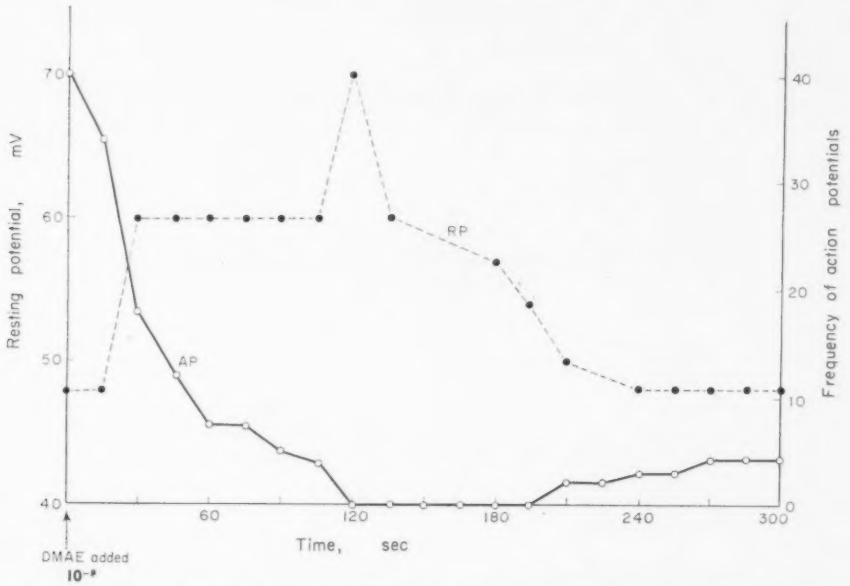

FIG. 6. The action of dimethyl-amino-ethanol (DMAE). DMAE caused an increase in the resting potential (●) and a decrease in the frequency of the action potentials (○).

Soaking the preparation in 10^{-5} g/ml of physostigmine potentiated the action to ACh so that the preparations became approximately 100 times more sensitive. On the other hand, addition of 2×10^{-7} g/ml atropine had no effect either on the spontaneous activity or on the sensitivity to ACh.

ACh, besides causing a change in the frequency of spontaneously firing cells, could sometimes also initiate activity in resting cells. On the other hand, a certain number of neurones were found that were spontaneously active but which were unaffected by the addition of ACh at physiological levels.

2. *Dimethyl-amino-ethanol* (*DMAE*). Dimethyl-amino-ethanol has been suggested as a possible natural precursor of choline (Pepev *et al.*, 1960). We did not try the action of choline on the preparation because we thought that choline would be produced after the esterase had acted on the acetylcholine. We found that in general, at physiological concentrations, DMAE tended to inhibit the spontaneous activity of the nerve cells. Fig. 6 shows the effect of adding 10^{-8} g/ml of DMAE to a spontaneously active nerve cell. The frequency of activity fell

from 40 per 15 sec to zero. At the same time the resting potential showed a hyperpolarization from 45 to 70 mV. As the resting potential recovered back to its initial level, so the frequency of action potentials increased. Higher concentrations

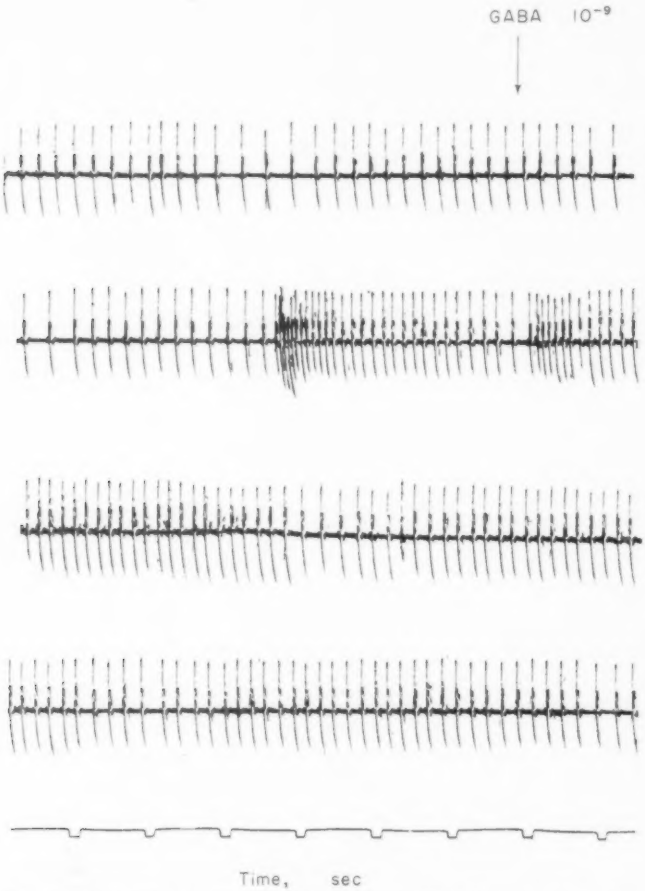

FIG. 7. The action of γ-aminobutyric acid (GABA). The above pen recording shows a continuous recording from a single unit. GABA at a concentration of 10^{-9} g/ml was added to the nerve cell and caused an increase in the frequency of the action potentials.

of DMAE (10^{-4}, 10^{-3}, 10^{-2}) tended to depolarize completely the membrane and maintain the resting potential at zero. This was the only drug, apart from acetylcholine and adrenaline, that caused complete depolarization.

A few nerve cells showed slight acceleration following addition of DMAE, the threshold concentration being 10^{-8}. Other nerve cells were quite unaffected by DMAE at physiological levels.

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3. *Glutamic acid.* Glutamic acid appeared to act on some neurones by accelerating them and on others by inhibition. The threshold for acceleration was 10^{-7} g/ml whilst that for inhibition was 10^{-10}. The inhibitory reaction occurred

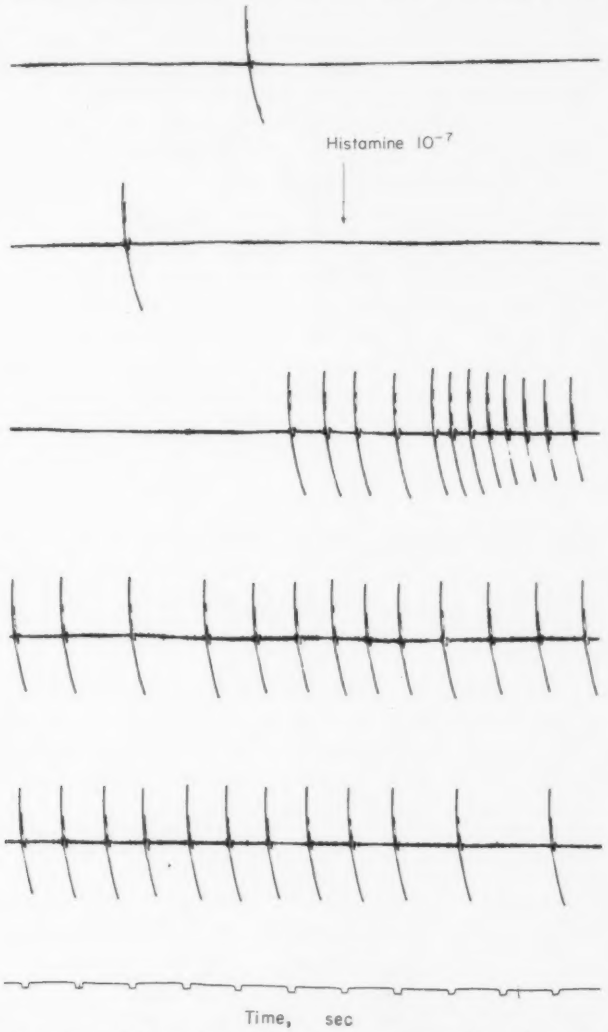

FIG. 8. The action of histamine. The above pen recording shows a continuous recording from a single unit. Histamine at a concentration of 10^{-7} g/ml caused an increase in the frequency of the action potentials.

within 5–10 sec after adding the glutamic acid, but the acceleratory effect was only noticeable about 1 min after application. Glutamic acid did not elicit activity from a resting cell.

4. *γ-Aminobutyric acid* (GABA). The overall effect of GABA was acceleration though it did cause inhibition to some neurones. The threshold for both acceleration and inhibition was 10^{-10} g/ml, though it was more usual for the effect to come in at 10^{-8}. Fig. 7 shows the effect of adding 10^{-9} GABA on a spontaneous neurone; the activity showed a marked increase 5 sec after the drug had been added. GABA did not bring the resting cell into activity.

5. 5-*Hydroxytryptamine* (5-HT). 5-HT caused both inhibition and acceleration of the spontaneous activity, the effect depending upon the cell under observation and the concentration of 5-HT used. In general the overall effect of 5-HT was acceleration. The reaction occurred within 5–10 sec of adding the 5-HT. The threshold for acceleration was 10^{-9} g/ml. The threshold for inhibition was 10^{-8} g/ml. 5-HT did not bring the resting neurone into activity.

6. *Histamine hydrochloride*. Histamine can cause acceleration or inhibition of the spontaneous activity depending on the type of neurone and the concentration of the drug. In general the type of neurone was more important. The reaction took place 5–10 sec after adding the histamine. The threshold for acceleration was 10^{-9} g/ml whilst that for inhibition was 10^{-8}. An accelerator cell would also be stimulated by higher concentrations of histamine. Fig. 8 shows the effect of adding 10^{-7} to an accelerator cell. This cell was also stimulated by 10^{-6} and 10^{-5}. Silent cells were often stimulated into activity by the addition of 10^{-6} histamine. Inhibitor cells could be affected by quite dilute solutions, but it is of interest to note that some cells were inhibited by 10^{-8} but stimulated by 10^{-6}. The inhibitory effect of histamine diminished with repeated application, thus successive applications of histamine at 10^{-5} to an inhibitory neurone caused inhibition of activity for $4\frac{1}{4}$ min the first time, $2\frac{3}{4}$ min the second time and 15 sec the third time. Histamine has been detected in the snail brain.

7. *Nor-adrenaline*. The overall effect of nor-adrenaline was an inhibitory one, though some neurones reacted by accelerating. The threshold for acceleration and inhibition was 10^{-9} g/ml. There was a general tendency for the response to nor-adrenaline to be a delayed one, the effect occurring a minute or so after addition of the drug. Nor-adrenaline did not affect the resting cell.

8. *Adrenaline*. Adrenaline affected the nerve cells in the main by causing an acceleration of the activity. On the other hand, a few cells did react by being inhibited. The threshold for both acceleration and inhibition was 10^{-7} g/ml. The more concentrated solutions of adrenaline tended to cause a proportional increase in the activity. This is illustrated in Fig. 9 where the maximum rate of action potentials is plotted against the added concentration of adrenaline. There was a linear relationship. Though adrenaline did cause an acceleration and a depolarization of the potential, it did not reduce the resting potential to zero even at high concentrations.

9. *Dopamine* (*3-hydroxytyramine*). The overall physiological effect of dopamine was inhibition. It did cause acceleration to some cells, the threshold for acceleration was 10^{-10} g/ml and that for inhibition 10^{-11}, though the usual threshold was 10^{-7} for acceleration and 10^{-9} for inhibition. Dopamine affected the resting

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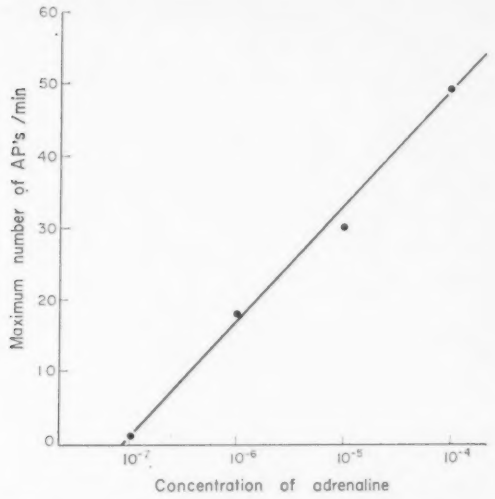

FIG. 9. The action of adrenaline. In this graph the maximum number of action potentials per minute is plotted against the added concentration of adrenaline. All data came from the same preparation. The higher concentrations of adrenaline caused a higher frequency of action potentials.

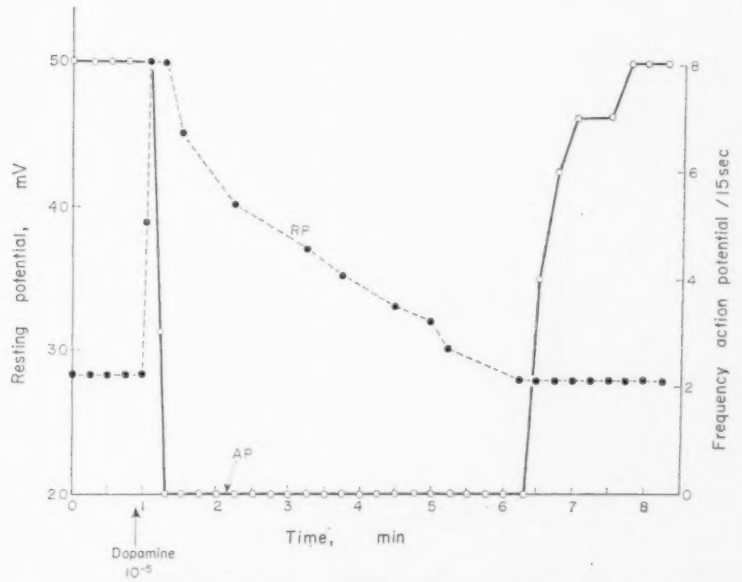

FIG. 10. The action of dopamine. Dopamine caused a sudden increase in the resting potential (●) and a decrease in the frequency of the action potentials (○). As the resting potential returned to the normal level, so the frequency of action potentials returned back to normal.

potential for the cells as is illustrated in Fig. 10. The addition of dopamine 10^{-5} caused a decrease in the frequency of the action potentials from 50 per 15 sec to zero. At the same time it hyperpolarized the membrane from 28 to 50 mV. As the

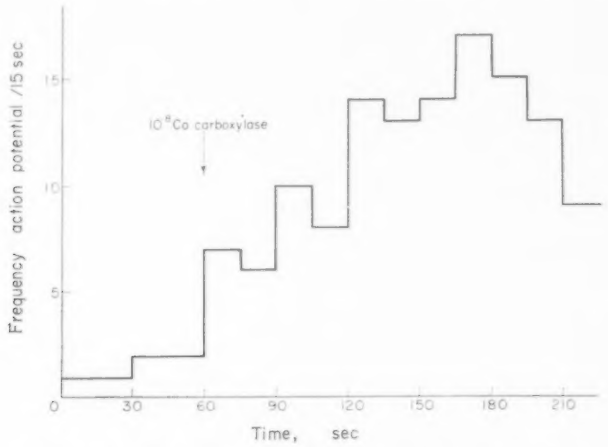

FIG. 11. The action of cocarboxylase. Addition of cocarboxylase caused an increase in the frequency of the action potentials.

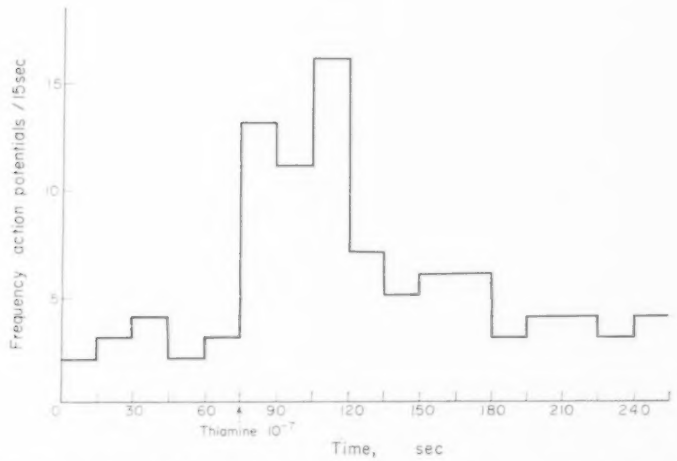

FIG. 12. The action of thiamine. Addition of thiamine caused an increase in the frequency of the action potentials.

resting potential slowly decreased to its original value, so the action potentials increased in frequency back to their original level.

10. DL-*phenylalanine*. The addition of phenylalanine to spontaneously active neurones tended to decrease their activity. The threshold concentration was

10^{-8} g/ml. The drug tended to cause a hyperpolarization of the resting potential and it could protect the membrane from complete permanent depolarization by 10^{-4} ACh. The response occurred within 5–10 sec of adding the drug. A few cells did not respond at all to the addition of phenylalanine.

11. *Cocarboxylase.* Cocarboxylase (thiamine pyrophosphate) elicited acceleration in some neurones, inhibition in others, and no effect in still others. The threshold for acceleration was 10^{-9} g/ml whilst that for inhibition was 10^{-8} g/ml. Fig. 11 shows the effect of adding 10^{-8} cocarboxylase to a preparation. The preparation showed an increase in activity much higher than anything recorded for that unit.

12. *Thiamine hydrochloride.* The general effect of thiamine was that of acceleration though quite a few cells were unaffected. The maximum threshold for acceleration was 10^{-8} g/ml though the average threshold was 10^{-7}. Fig. 12 shows the effect of adding 10^{-7} thiamine to a unit; the activity increased. No inhibition was noted following the addition of thiamine.

DISCUSSION

The snail preparation proved to be sensitive to a majority of the chemicals described in the previous section, the reactions occurring at physiological drug levels of 10^{-6} g/ml or less. This is perhaps to be expected if the results are to have any natural significance since the drugs were being applied directly to the nerve cell under observation; there was no connective tissue between the chemicals and the nerve to act as a diffusion barrier. Most of the drugs that had an effect on the preparation elicited their effect within 5–10 sec after addition. There were some exceptions to this, thus nor-adrenaline took a minute or so to obtain a reaction, and glutamic acid required over a minute for its acceleratory effect to be seen. The quick response to the addition of a drug, and the relationship between the resting potential and the frequency of the action potentials, indicate that it is most likely that the drug is acting directly on the cell under observation, and not via the nerve cells in the rest of the brain.

The drugs described usually had a triple effect on the nerve cells; they accelerated, they inhibited, and they had no effect! This result can be seen more clearly from Table 1 and it raises several problems. Are the results due to chance, experimental variability, concentration effects, or are they due to cellular differences? From our results it would appear that the last two of these explanations are correct. The most important variable would appear to be the cell under observation. Certain cells, no matter what concentration range was used, would only show inhibition of their activity following addition of the drug. Other cells would give acceleration if the drug was administered at low concentrations, and inhibition if the drug was added at a higher concentration. Other cells, still, were unaffected by the administration of the drug. The results obtained following administration of a given chemical were usually striking and determined, even if they were exactly opposite to those obtained in a previous experiment. These results make it imperative to know the precise histological localization of each cell

under observation before one can state with certainty the effect of a chemical on the nerve cell. This conclusion is not altogether unexpected. Bradley & Mollica (1958) working on single units in the mesencephalon and medulla of the decerebrate cat found that adrenaline accelerated some cells, inhibited others, and had no effect on still others. Acetylcholine on the other hand always had an effect though in some cases it was an acceleration and in other cases an inhibition. The most reasonable conclusion is that the nervous system shows heterogeneity in its reactions to chemicals; certain cells react in one manner whilst other cells react in another to the administration of a given chemical.

TABLE 1

Drug	No. of expts.	Threshold acceleration (g/ml)	Threshold inhibition (g/ml)	Overall effect
Acetylcholine	39	10^{-8}	10^{-7}	Acceleration
Adrenaline	19	10^{-7}	10^{-7}	Acceleration
Cocarboxylase	12	10^{-9}	10^{-8}	Both
Dimethyl-amino-ethanol	11	10^{-8}	10^{-6}	Inhibition
Dopamine	21	10^{-10}	10^{-11}	Inhibition
GABA	11	10^{-10}	10^{-10}	Acceleration
Glutamic acid	14	10^{-8}	10^{-10}	Both
Histamine HCl	15	10^{-9}	10^{-8}	Both
Nor-adrenaline	19	10^{-9}	10^{-9}	Both
5-HT	20	10^{-9}	10^{-9}	Acceleration
Phenylalanine	9		10^{-8}	Inhibition
Thiamine HCl	8	10^{-8}		Acceleration

The second problem concerns the evidence that any of the tested chemicals is a natural transmitter in the central nervous system of the snail. Let us briefly consider the possibility for each of the tested chemicals.

Acetylcholine. Acetylcholine acts on the snail nerves at a physiological level (10^{-8} g/ml). It has both a stimulatory and an inhibitory effect. A similar conclusion was drawn by Tauc & Gerschenfeld (1960) for the nerve cells of *Helix pomatia*. They found that 10^{-8} stimulated and 10^{-5} inhibited, and have put forward the interesting suggestion that each nerve cell was capable of reacting to ACh, the stimulation or inhibition depending on the resting potential of the cell. If the potential was above 50 mV, then ACh stimulated; if it was below 50 mV then ACh inhibited. The critical voltage (50 mV) varied from cell to cell.

We have found no clear relationship between a cell's resting potential and the manner in which it reacts to acetylcholine. Thus, cells with resting potentials varying from -50 to -10 mV have had their spontaneous activity inhibited by the addition of acetylcholine. Other cells with resting potentials varying over the same range have had their spontaneous activity increased by the addition of acetylcholine. Yet a third group of cells with the same range of resting potentials have been unaffected by acetylcholine.

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Other workers have found ACh to have an effect on gastropod nerves.

Duncan (1961) showed that ACh at 2×10^{-5} stimulated the isolated pedal nerves of the snail *Physa*.

We have found an acetylcholine-like substance present in *Helix* brain. When tested on the rat jejunum it gave a concentration equivalent to 5–10 μg/g of brain tissue. Chromatographic analysis also indicated an ACh-like substance to be present. Choline esterases are known to be present in the blood (Augustinsson, 1946) and we have shown the presence of cholinesterase histochemically in snail nerve cells. Thus, within the snail brain there is an ACh-like substance, the nerve cells are sensitive to ACh at levels lower than those found in the whole brain, and their sensitivity is increased by eserine. The evidence is reasonably strong that ACh could be a natural CNS transmitter in the snail.

Dimethyl-amino-ethanol. Dimethyl-amino-ethanol in general had an inhibitory effect (10^{-6}). Addition of DMAE did not have an immediate effect on the neurone; there was normally a delay of 60–100 sec before a response occurred. The hyperpolarization of the cells and the inhibition of the activity resemble the inhibitory action of acetylcholine and it is possible that DMAE might be converted into ACh though there is, at present, no definite evidence that can support this view.

γ-Aminobutyric acid (GABA). It was suggested by Bazemore *et al.* (1957) that GABA was the material responsible for the physiological action of the Factor I extract from the mammalian brain. More recently Florey & Chapman (1961) have shown that Factor I and GABA are not synonymous since Factor I is a much more powerful inhibitor than GABA. Nevertheless, GABA has a most marked inhibitory action on the crustacean stretch receptor.

We found that the overall effect of GABA was a stimulatory one though a few units did respond by decreasing their activity. The effect of GABA is, in general, a temporary one, and the accelerated neurone soon returned back to its initial firing rate. Hirchar (1960b) found that GABA at concentrations from 10^{-10} to 10^{-2} M had no effect on the spontaneous activity of the nerves in the fifth abdominal ganglion of the crayfish *Orconectes virilis*, which may indicate that the central neurones of the crayfish may react differently from the peripheral nerves in the reaction to GABA.

Glutamic acid. Glutamic acid is considered to be the metabolic precursor of GABA. It also plays a role in the metabolism of the nervous system (Strecker, 1957). We found that the nerve cells were sensitive to the addition of glutamic acid (inhibition threshold, 10^{-10}; acceleration threshold, 10^{-8}). There was, however, no overall effect of this drug, the acceleratory effect was equally common to the inhibitory effect. It was noticeable, however, that the inhibition was in general irreversible—the cells did not recover. With an inhibitor such as dopamine, on the other hand, the overall effect was that inhibition and the results were reversible. We have detected glutamic acid in the brain of the snail.

Phenylalanine. We investigated this compound for two reasons; first, because it has been found that *Helix* hearts kept in saline, enriched with phenylalanine, will beat for 24 days (Jullien *et al.*, 1956). Secondly, phenylalanine is a possible intermediate in the formation of dopamine. The administration of C^{14} phenylalanine

to rats resulted in the recovery of radioactive adrenaline (Gurin & Delluva, 1947). We found that the effect of phenylalanine at 10^{-8} was an inhibitory one, and that in particular it could repolarize the nerve membrane and protect it against the action of strong concentration (10^{-5}) of acetylcholine.

Dopamine. The overall effect of dopamine was to inhibit the activity of the snail cells (maximum threshold, 10^{-11}). The majority of the responses were delayed ones, i.e. they took over a minute to have an effect and thus it is possible that cells other than the recorded ones might be affected by dopamine. Dopamine hyperpolarized the nerve membrane and in doing so it was the most effective of all the drugs tested. Ostlund (1954) was unable to find dopamine in the molluscs though he did find an unidentified catechol amine which he called "catechol 4". Price (unpublished) has isolated a material from the brain of gastropods which gives a spot at the same R_f as dopamine, and which, like dopamine, inhibited the isolated frog heart. Thus, there is a fair possibility that dopamine exists in the snail brain. It has a possible role as an inhibitory compound in the snail brain.

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Adrenaline and nor-adrenaline. The preparations were more sensitive to nor-adrenaline than adrenaline. The effects of nor-adrenaline were mixed; it stimulated some neurones and inhibited others. Adrenaline, on the other hand, had mainly an excitatory effect. We have not been able to detect adrenaline or nor-adrenaline in chromatograms of snail material and think it unlikely that these substances occur naturally in the animal.

5-*Hydroxytryptamine.* 5-HT has been suggested as a cardio-regulator in molluscs by Bacq *et al.* (1952) and Welsh (1953). Its distribution in the invertebrates has been investigated by Welsh & Moorhead (1960), and they have found quantities in the gastropods ranging from 2·6 μg/g brain tissue in *Polynices heros* to 11·0 μg/g brain tissue in *Melongena.* We have, so far, been unable to find any valid concentrations of 5-HT in our chromatograms of the snail-brain extract (Kerkut & Laverack, 1960). Our recent investigations have indicated that if 5-HT is present, it will be in concentration less than 0·5 μg/g. On the other hand, we have found that the overall effect of 5-HT is to accelerate the neurones, the drug being effective at a concentration of 10^{-9} g/ml.

Thiamine hydrochloride and cocarboxylase. Von Muralt (1945, 1958) suggested that thiamine compounds played a double role in the nervous system. Cocarboxylase (thiamine pyrophosphate) was important in metabolic systems whilst thiamine was more important in the transmission of the nerve impulse. We found that cocarboxylase could both excite and inhibit and which ever it did was effective in reasonably high dilutions (10^{-8} and 10^{-9} g/ml). Thiamine, on the other hand, was an excitant (10^{-8}). This tends to agree with von Muralt's finding that more thiamine was present in excited nerves than non-excited nerves. Similarly, Petropulos (1960) found that antithiamine compounds caused a hyperpolarization of the nerves of *Rana esculenta.* Augustinsson (1946, 1948) has described an enzyme in *Helix* dart sac and blood which will hydrolyse acetylthiamine.

Histamine. Histamine could cause both excitement and inhibition depending on the cell under investigation. The threshold for acceleration was 10^{-9} and for

inhibition 10^{-8}. The stimulatory effect soon faded, but the inhibitory response was more lasting and the neurone in general failed to return to its initial rate of discharge. Preliminary investigations indicate that histamine may be present in the snail ganglia.

Thus, of the chemicals tested, acetylcholine, dopamine, glutamic acid and histamine have been found in the snail brain; 5-hydroxytryptamine, thiamine, cocarboxylase, dimethyl-amino-ethanol, γ-aminobutyric acid and phenylalanine are possibly present. We doubt whether adrenaline and nor-adrenaline are present. It is difficult to conclude whether any of the above chemicals are natural excitants and inhibitors of the nervous system because the results so far merely indicate that excitation and inhibition can occur. The fact that all these chemicals have an effect at physiological concentrations is of interest, and the further fact that some of them are known to be present in the snail strengthens their claim as natural neuro-chemical transmitters. The strongest case, so far, is that for acetylcholine which also has an esterase present. Dopamine is a possible inhibitory substance. However, it is clear that further work on specific nerve cells whose anatomical relationships are well known and precisely defined will be necessary before one can conclude the extent to which any given chemical acts as a neurotransmitter and also the extent to which chemical heterogeneity exists in the central nervous system.

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SOME SPECTROPHOTOMETRIC STUDIES ON RESPIRING PARTICLES ISOLATED FROM THE HEPATOPANCREAS OF *CARCINUS MAENAS*

R. B. BEECHEY

Department of Physiology and Biochemistry, Southampton University

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Abstract—(1) The preparation of phosphorylating respiring particles from the hepatopancreas of *Carcinus maenas* is described.

(2) Two methods were used to find the difference spectrum of the particles reduced by succinate.

(3) The general difference spectrum indicates the presence within these particles of cytochromes *a*, a_3, *b*, *c* and c_1, flavoproteins and pyridine nucleotides. Two unusual features of this spectrum are two small peaks at 595–600 mμ and around 612 mμ. The former may be ascribed to the α-band of reduced a_3.

(4) The cytochromes are present in approximately equimolar concentrations. The concentrations of the flavoproteins and pyridine nucleotides are respectively 3 and 19 times that of the cytochromes.

(5) The speed of the reduction of the cytochrome and flavoprotein pigments suggests that these are components of the respiratory chain concerned with the oxidation of succinate. The pyridine nucleotides are reduced too slowly to be involved directly in the oxidation of succinate.

(6) Diphasic reversals at 445 mμ indicate the presence of at least two pigments absorbing light at this wavelength.

(7) The composition of the respiratory chain in these respiring particles is discussed in relation to the composition of the respiratory chain of other animals.

INTRODUCTION

In his classical studies Keilin (1925) presented evidence for the presence of respiratory chains containing cytochrome pigments in widely differing groups of invertebrate animals. However, the present concept of the respiratory chain is a complex of cytochromes, flavoproteins, pyridine nucleotides, linked proteins and a number of non-porphyrin electron carriers situated in the mitochondrion. This conclusion is based mainly on results obtained using mammalian tissues (Slater, 1958). The results of the few investigations on tissues from other groups of organisms have shown that variations from the mammalian pattern of respiratory chain do occur, e.g. the cytochrome systems of bacteria (Smith, 1954), and the presence of cytochrome *h* in certain invertebrate tissues (Keilin, 1956).

Ghiretti and his co-workers have studied the respiratory chains present in the muscle of the molluscs, *Aplysia depilans* and *A. limacine* (Ghiretti *et al.*, 1959) and *Octopus vulgaris* (Ghiretti-Magaldi *et al.*, 1957, 1958). They showed that cytochromes *a*, a_3, *b*, *c*, c_1 and flavoprotein were present in the *Aplysia* muscle preparation and cytochrome *h* in the hepatopancreas. The *Octopus* muscle

preparation contained cytochromes *a*, a_3, *b*, *c*, flavoproteins and pyridine nucleotide-linked proteins. Thus, while the respiratory chain of these species broadly resembles that of mammals, detailed studies have revealed that the properties of purified cytochrome *c* from *Sepia officinalis* differ greatly from those of purified mammalian cytochrome *c* (Ghiretti-Magaldi & Ghiretti, 1958).

In a recent study Tappel (1960) showed that the difference spectrum of the muscles of some crustaceans indicated the presence of cytochromes in that tissue. The muscle of *Callinectes sapidus* appeared to contain cytochromes *a*, a_3, *b*, *c* and c_1, whilst no cytochrome *c* could be detected in *Homarus americanus*, and the shrimp *Pandulus* sp. contained a second member of the cytochrome *b* group. In the bivalve molluscs, *Crassostrea virginica*, *Mercenaria mercenaria*, *Pectin irradians* and *P. magellanicus*, however, the concentration of cytochromes *a* and a_3 was very low.

This paper deals with the respiratory chain in particles isolated from the hepatopancreas of the crustacean *Carcinus maenas*. Evidence is presented to show that these particles contain cytochromes *a*, a_3, *b*, *c*, c_1, flavoproteins and pyridine nucleotide-linked proteins. Data on the kinetics of the change of these pigments from the oxidized to the reduced states are also given. A summary of these results has been reported briefly elsewhere (Beechey, 1961).

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METHODS

Preparation of the respiring particles

The hepatopancreas was removed from the specimens of *Carcinus maenas* and placed in ice-cold 0·25 M sucrose, to cool the tissue and wash away any blood surrounding the tissue. (The pH of the sucrose solution had been previously adjusted to approximately 7·4 with dilute potassium hydroxide, using bromothymol blue as an external indicator.) When sufficient material had accumulated (usually fifteen to twenty crabs, carapace width 5–6 cm, were used) the 0·25 M sucrose was drained off. The tissue was then homogenized with an equal volume of fresh 0·25 M sucrose in a hand homogenizer, the approximate clearance between the plunger and the tube was 0·4 mm. Four or five insertions of the plunger were sufficient to disrupt most of the tissue. The homogenate was diluted with its own volume of 0·25 M sucrose in an MSE major centrifuge at 1°C for 5 min at 800–900 g (bottom of the tube). The supernatant fluid was decanted into clean centrifuge tubes and recentrifuged for 10 min at 8500 g. The supernatant fluid and any very loosely packed material was then rejected. The pellet had three layers. The uppermost comprised a considerable amount of loosely packed dark buff material; the middle, a smaller amount of a more firmly packed light buff-coloured material and the lowest layer, a small amount of black material tightly packed at the bottom of the tube. The two upper layers were suspended in 1–2 ml of 0·25 M sucrose by alternate sucking and blowing through a Pasteur pipette (Holton, 1955). The thick suspension was then diluted with 35 ml of 0·25 M sucrose, recentrifuged at 8500 g for 10 min and the supernatant was rejected. Approximately 5 ml of 0·25 M sucrose was carefully added to the tube.

which was gently agitated by hand in order to wash off the less firmly packed dark buff material. The washing was repeated until the underlying layer could be seen clearly. The exposed light buff material was then suspended in approximately 1 ml of 0·25 M sucrose or the succinate suspension medium, care being taken to avoid disturbing any of the firmly packed black material which was at the bottom of the tube. This concentrated suspension (protein content 15–20 mg/ml) was used in the experiments described. All stages of the preparation, which took 90–100 min to complete, were carried out at 0–4°C.

This method of preparation was evolved in collaboration with Mr. B. D. Thompson and Dr. K. A. Munday of this department and further details will appear in a later publication.

Suspension media

For experimental purposes, 0·5 ml of the final concentrated suspension of respiring particles was diluted with one of the following solutions. The succinate suspension medium contained 5 mM sodium succinate, 5 mM sodium, potassium phosphates pH 7·4, 1 mM tripotassium adenosine triphosphate, 5 mM magnesium chloride and 0·2 M sucrose. The "no substrate" suspension medium had the same composition save that the sodium succinate was omitted and the sucrose concentration was adjusted to 0·22 M. The final tonicity of both these suspension media was 0·25 osM.

Experimental procedures

Two procedures were used: the reversal and direct difference procedures. In experiments where only the reversal technique was used the final suspension of the respiring particles was made in the succinate suspension medium. When the direct difference technique was employed, the final suspension was made in 0·25 M sucrose. All experiments were carried out at room temperature, 18–20°C. An optical cell of 1 cm light path was used in a Zeiss Spectrophotometer PMQ II, with a double monochromator. Glass or quartz prisms were employed as appropriate. The optical cells were placed as close as possible to the photomultiplier light detector, in order to minimize losses due to the scattering of light by the particle suspension.

The reversal technique

This is basically the procedure first described by Holton (1955). The wavelength and slit adjustments were set as required. The extinction scale reading of the spectrophotometer was then set at an arbitrary figure, usually between 0·05 and 0·15, by adjusting the amplification of the photocurrent from the photomultiplier light detector. During these manipulations, the respiratory pigments were in a reduced state. Oxygen saturated with water vapour was then gently bubbled through the particle suspension for a measured time; this was 5–20 sec, depending upon the respiratory activity of the particles. Measurements of the extinction of the particles were then made every 6 or 12 sec. During the aerobic

steady state, the extinction was almost constant. (In this state the respiratory pigments are oxidized, oxygen being in excess.) When the oxygen concentration became the limiting factor, a phase followed in which rapid changes in extinction occurred. The rate of change of extinction then declined leading into the anaerobic steady state. (At this point the pigments are reduced and the oxygen concentration is effectively zero.) The spectrophotometer was then checked for zero drift and the whole reversal procedure was repeated at a new wavelength. The measured extinction values were plotted against time (see Fig. 5) and the difference between the extinction values of the suspension in the oxidized and reduced states measured. This difference is referred to as ΔE_λ. The difference spectrum of the particles was obtained by plotting ΔE_λ against wavelength.

The wavelength at which the greatest ΔE_λ changes occurred was 445 mμ. Hence the kinetics at 445 mμ were used as a reference to detect changes in the behaviour of the respiratory pigments. In every reversal experiment the kinetics of the reversals at 445 mμ were measured at the beginning of the experiment and at every sixth reversal. The experiment was abandoned when the reversals at 445 mμ showed successive abnormalities. During the constant phase of the 445 mμ reversals (see later) the standard deviation for changes in ΔE_{445} was never greater than 0·004 extinction units and was usually less than 0·003 extinction units (see Fig. 5). The measurement of the time of oxygenation was done for two reasons: firstly it avoided an excessively long aerobic steady state caused by over oxygenation, and secondly it enabled a rough check to be made of the respiratory activity of the suspension. If this declined quickly, as indicated by a rapid lengthening of the aerobic steady state, then the experiment was concluded.

Direct difference experiment technique

In this method, the difference spectrum is obtained by measuring the variations with wavelength in the extinction of a reduced sample of respiring particles using an oxidized sample as an optical blank.

The final concentrated suspension of the particles was made in 0·25 M sucrose. Two samples of this suspension were diluted, one with the succinate suspension medium and the other with the no-substrate suspension medium. A reversal at 445 mμ was carried out on the succinate suspension to ensure that the pigments were reduced. Oxygen saturated with water vapour was then gently bubbled through the no-substrate suspension for 0·5–2 min in order to oxidize the respiratory pigments. The variation of the extinction of the reduced sample with wavelength was then measured using the oxidized sample as a blank. The extinction of the oxidized sample at 445 mμ was measured before and after each wavelength scan to ensure that the pigments had remained oxidized during the scan.

Designation of the absorption bands

The nomenclature of Keilin & Hartree (1939) has been used for this purpose. Thus $b''\alpha$ refers to the α-band of reduced cytochrome b'' and $b'''\alpha$ refers to the α-band of the oxidized band of cytochrome b'''.

RESULTS

General properties of the hepatopancreas respiring particles

When suspended in the succinate medium the particles show a metabolic quotient of 30–40 μl O_2/hr/mg protein at 25°C and a mean phosphorus/oxygen (P/O) ratio of $1{\cdot}6 \pm 0{\cdot}2$ (ten determinations). This last result was obtained by using an inorganic phosphate uptake method with a hexokinase–glucose phosphate trap. Oxygen consumption was measured manometrically and protein content by the Biuret method.

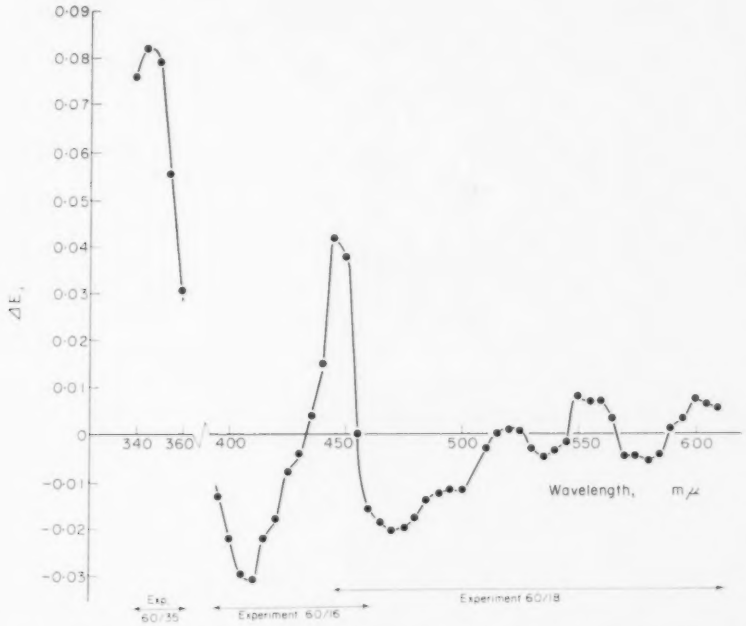

FIG. 1. The difference spectrum of the hepatopancreas respiring particles reduced by succinate. Half-band widths 3·70 mμ at 340 mμ, 2·70 mμ at 360 mμ, 0·46 mμ at 400 mμ, 0·57 mμ at 450 mμ, 0·54 mμ at 500 mμ, 0·38 mμ at 550 mμ, 0·51 mμ at 600 mμ. Reversal experiments.

Difference spectrum

The difference spectrum of the respiring particles in the range 340–620 mμ is shown in Fig. 1. This is a composite figure drawn from the results of three experiments. The general pattern of light absorption suggests that the respiring particles contain pyridine nucleotide-linked proteins, flavoproteins and cytochromes of the *a*, *b* and *c* groups.

The large absorption band with a peak at 445 mμ and the smaller band about 600–610 mμ respectively correspond to the $(a+a_3)$ γ- and α-bands found in material from mammalian sources. (In the following paragraphs the reference

bands quoted are those of mammalian respiratory pigments.) The presence of cytochrome *b* is indicated by the absorption of light at 560–565 mμ and the slight asymmetry in the 425–435 mμ region. These features correspond to the *b''*α- and *b''*γ-bands. The weak *b''*β-band at 530 mμ appears to be masked by the absorption

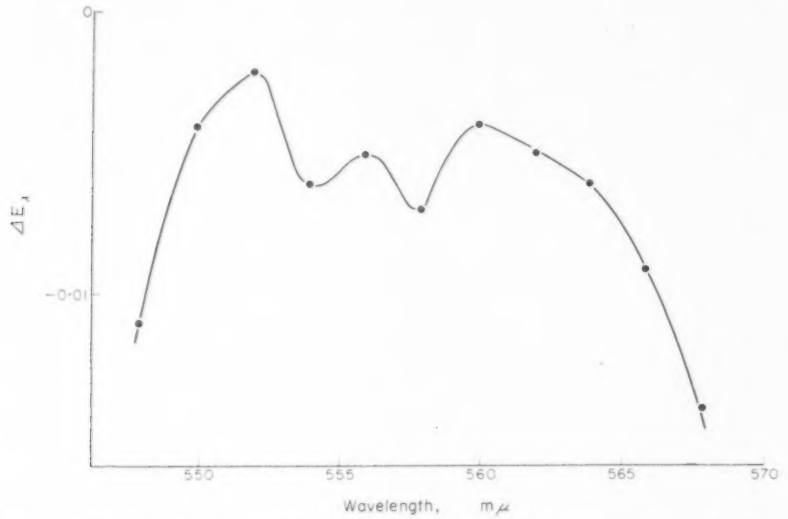

FIG. 2. Detailed difference spectrum of the hepatopancreas respiring particles reduced by succinate. Half-band widths 0·76 mμ at 550 mμ and 0·86 mμ at 570 mμ. A reversal experiment.

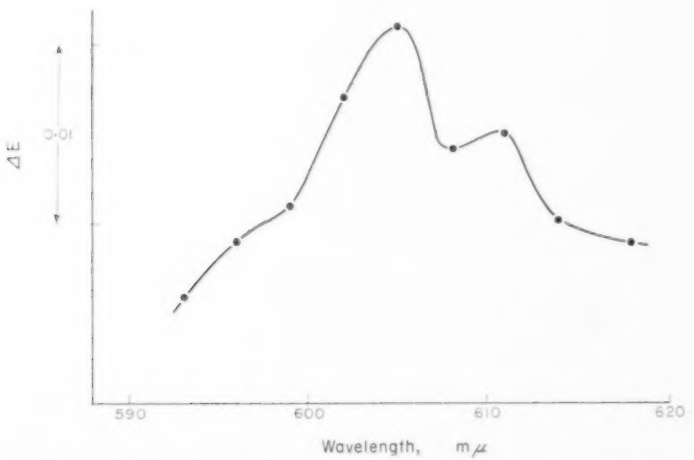

FIG. 3. Detailed difference spectrum of the hepatopancreas respiring particles reduced by succinate. Half-band widths 0·98 mμ at 595 mμ, 1·14 mμ at 605 mμ, 1·46 mμ at 615 mμ. A direct difference spectrum experiment.

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of oxidized cytochrome *c* in this region. The presence in these particles of at least one of the cytochrome *c* group is suggested by the absorption bands at 550–560 mμ, 515–525 mμ and the asymmetry between 415 and 425 mμ. The absorption of light in these regions corresponds respectively to the $(c+c_1)''$ α-, β- and γ-bands. The large trough between 455–515 mμ is probably due to two

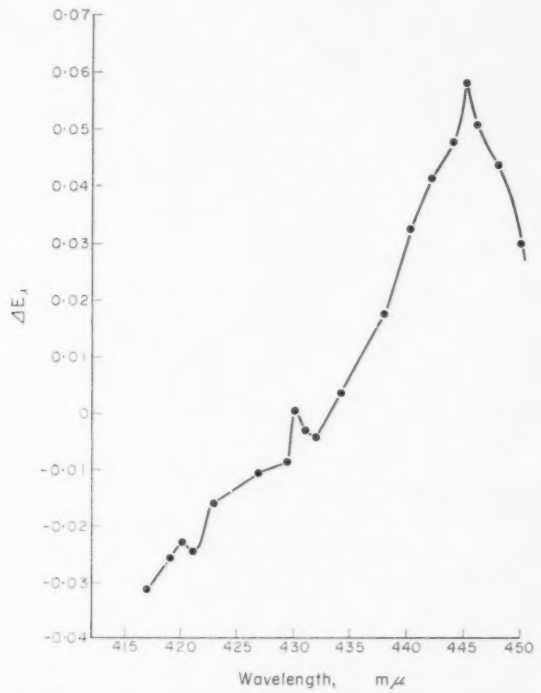

FIG. 4. Detailed difference spectrum of the hepatopancreas respiring particles reduced by succinate. Half-band widths 0·58 mμ at 450 mμ, 0·44 mμ at 420 mμ. A reversal experiment.

complex components: (1) the absorption of light by the oxidized flavoprotein constituents of the respiratory chain; (2) contributions from the oxidized cytochromes *a*, a_3, *b*, *c* and c_1. Similarly, the trough between 395 and 433 mμ is probably the resultant of the absorbances of the γ-bands of oxidized and reduced cytochromes *b*, *c* and c_1 and the oxidized cytochromes *a* and a_3.

The absorption band with a peak at 345 mμ (see Fig. 1) indicates that pyridine nucleotides are present in these respiring particles. The particles have a high intrinsic capacity to absorb ultra-violet light. This necessitated working with wide slit widths and hence large half-band widths, which obviated the precise localization of peaks in this region.

The spectral interval 360–395 mμ is not recorded in Fig. 1. However, in this range the relationship between the extinction and wavelength is almost linear.

The difference spectrum was investigated in detail in the following regions, 545–570 mμ, 590–620 mμ and 417–450 mμ. Figs. 2–4 show the results of such experiments.

The 545–570 mμ *region*

The difference spectrum in this region usually showed three peaks (see Fig. 2). The peak which lay between 549 and 552 mμ can be ascribed to the $c''\alpha$-band and that which appeared in the range 560–564 mμ is the $b''\alpha$-band. The smaller peak lying between 554 and 556 mμ is probably the $c_1''\alpha$-band. Only occasionally was it impossible to detect this peak. For technical reasons, readings were taken at 2 mμ intervals which partly accounts for the slight uncertainty in ascribing an exact wavelength to each peak absorption.

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The 590–620 mμ *region*

The main feature of the difference spectrum in this region was an absorption band with a peak at 605 mμ (see Fig. 3). In most experiments shoulders could be detected at 595–600 mμ and around 610 mμ. The peak at 605 mμ corresponds to the $a''\alpha$-band and the shoulder at 595–600 mμ probably represents the $a_3''\alpha$-band (Lundegårdh, 1957), but the shoulders around 610 mμ cannot be ascribed to any cytochrome pigment of metazoan origin.

The 417–450 mμ *region*

It can be seen that in Fig. 4 the maximum absorbance is at 445 mμ, which coincides with the $(a+a_3)''\gamma$-band. In addition to the major peak at 445 mμ, subsidiary peaks at 430 mμ and 420 mμ can be seen, which coincide respectively with the $b''\gamma$- and $(c+c_1)''\gamma$-bands. These results have been confirmed by direct difference spectrum experiments, using freshly prepared particles.

The kinetics of extinction changes due to the reduction of respiratory pigments

The use of a single-beam spectrophotometer, for the type of experiments reported here, introduces the problem of adequate control. The main difficulty is to assess what proportion of the measured extinction changes are caused by changes in the size of particles (Cleland, 1952) as opposed to those due to changes in the oxidation state of the respiratory pigments. The relative constancy of the extinction value in the aerobic and anaerobic states suggests that no changes in the size of the particles occur in these states, but with the present techniques it is impossible to detect changes in the particle form which might occur during the phase of rapid extinction changes.

At the majority of wavelengths studied, the reversals followed a smooth sigmoid type of curve, i.e. they were monophasic, see Fig. 5, reversals 2–5. Such behaviour is to be expected if the extinction changes are caused by a single pigment or a group of pigments reacting with similar velocities. At certain wavelengths, however,

the reversals were characteristically diphasic, i.e. the rate of change of extinction with time altered rapidly at some stage in the reversal, see Fig. 5, reversals 1, 6, 7.

The kinetics of reversals at 445 mμ

In all reversal experiments the behaviour at 445 mμ was used as a reference point (see Methods section) and consequently a considerable amount of information has accumulated concerning the behaviour at this wavelength.

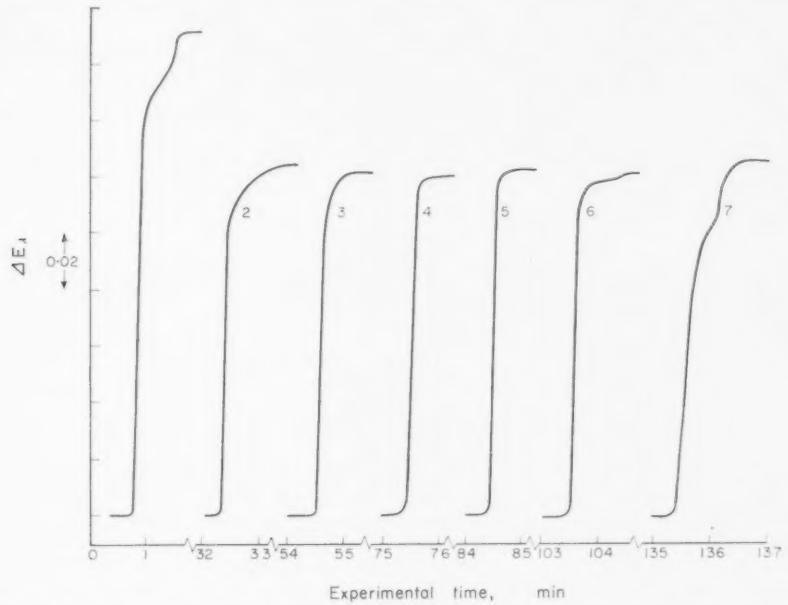

FIG. 5. The reproducibility of reversals at 445 mμ in a single experiment. (Reversals at other wavelengths were made in the intervals between measurements at 445 mμ.) Succinate as substrate. Half-band width 0·57 mμ.

During the course of an experiment the magnitude of most of the 445 mμ reversals was constant. However, it was noted that in the very early stages of many experiments, the ΔE_{445} was significantly greater than the subsequent constant ΔE_{445} (see Fig. 5). The cause of this rapid decrease of ΔE_{445} might be due to the degeneration of the respiratory chain from a phosphorylating to a non-phosphorylating mechanism.

Eventually, during the course of an experiment the constant ΔE_{445} reversals gave way to atypical reversals which were characterized either by a further decrease in magnitude of the ΔE_{445} or a marked increase in the time taken to change from the aerobic to the anaerobic steady state. On some occasions a preparation showed simultaneously both types of change. These changes were regarded as signs of further degeneration of the respiring particles and the experiment was concluded.

Approximately one quarter of all the reversals at 445 mμ were diphasic. Reversals 1 and 6 of Fig. 5 are typical examples of the diphasic 445 mμ reversal. Reversal 7 of Fig. 5 is an extreme example. Such examples were not recorded frequently. The factors causing the reversible changes between monophasic and diphasic kinetics in the course of a single experiment have not been established. However, the diphasic reversals suggest the presence of at least two respiratory pigments absorbing light at 445 mμ in the reduced state. The obvious candidates are cytochromes *a* and a_3.

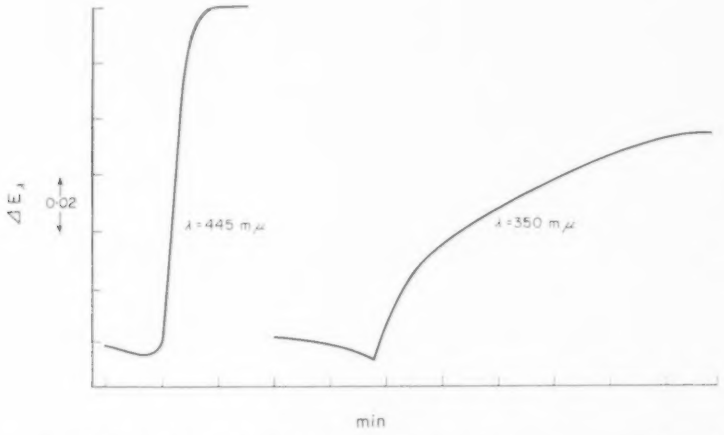

FIG. 6. The variation of extinction with time at 445 mμ and 350 mμ. A reversal at 445 mμ performed immediately after the 350 mμ reversal was identical with the 445 mμ reversal illustrated here. Succinate as substrate. Half-band width 1·29 mμ at 445 mμ and 3·28 mμ at 350 mμ

Reversals around 340 mμ

The most striking feature of reversals in this region was the slow rate of conversion from the oxidized to the reduced states compared with the rate at 445 mμ (see Fig. 6). This slow rate of reduction suggests that the pyridine nucleotides are not intermediates in the respiratory chain responsible for the oxidation of succinate. The electrons for the reduction of the pyridine nucleotides may come from the slow oxidation of an endogenous substrate or via the pyridine nucleotide–cytochrome *b* reductase system. The latter is made to reverse its action as the free energy potential of the reduced *b* pigments slowly increases to a level enabling the reduction of oxidized pyridine nucleotide.

DISCUSSION

The spectroscopic evidence presented here suggests that the hepatopancreas respiring particles contain pigments which correspond to the mammalian cytochromes *a*, a_3, *b*, *c* and c_1 as well as flavoproteins and pyridine nucleotides. (It is

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obvious that the separation and examination of the purified individual components is essential before their presence can be confirmed.) These pigments are also the constituents of the organized respiratory chains found in mammalian mitochondria (Slater, 1958; Chance & Baltscheffsky, 1958). It is tempting to assume that the hepatopancreas pigments interact with the same degree of specificity shown by the mammalian respiratory pigments, but no evidence has been adduced on this point.

The difference spectrum (see Fig. 1) is remarkably similar to that of the other phosphorylating particles (Chance & Williams, 1955a; Holton, 1955; Chance & Baltscheffsky, 1958). The detailed difference spectrum in the range 590–620 mμ (see Fig. 2) is of interest in that the a_3''α-band is present as a shoulder in the 595–600 mμ region. A similar feature was noted by Lundegårdh (1957) in the difference spectrum of yeast and cytochrome ($a+a_3$) preparation purified from heart muscle. This author calculated the peak of the a_3'' α-band to be at 598 mμ. The peak or shoulder around 610 mμ cannot yet be explained in terms of the behaviour of a respiratory pigment of metazoan origin. However, a cytochrome a_4 in *Acetobacter peroxydans* with a band at 612 mμ in the reduced form has been described by Chin (1952), but there is no evidence to suggest a connexion between these two phenomena.

The absence of interference caused by oxygen-carrying pigments of the blood is easily achieved by washing the exterior of the tissue. *Carcinus maenas* has an open blood circulatory system, there being no system of blood vessels within the tissues similar to that found in mammalian tissues.

Slater (1958) has suggested three criteria which must be satisfied before a compound may be considered a member of the respiratory chain. They are:

(1) It must be present in enzyme preparations in amounts commensurate with the enzyme activity. (In a later section of his review, Slater indicates that this criterion is satisfied if the component is present in amounts which are of the same order of magnitude as other components of the respiratory chain.)

(2) Its removal from the enzyme preparation either by extraction or combination with an inhibitor must result in inactivation and the activity must be restored by the addition of the substance or removal of the inhibitor.

(3) It must be shown to undergo oxidation and reduction during the operation of the enzyme system at a rate in agreement with the overall activity of the system.

The data listed in Table 1 show that the cytochromes a, a_3, b, $c+c_1$ are present in approximately equimolar concentrations. The flavoprotein content appears to be approximately 3 times and 19 times more than that of cytochrome a_3. These general proportions in no way contrast with the corresponding data given in Table 1 for rat heart sarcosomes (Chance & Baltscheffsky, 1958) and liver mitochondria (Chance & Williams, 1955a), two systems whose respiratory chains have been well characterized and are accepted as the classical type.

The low relative cytochrome b content of *Callinectes* and *Homarus* muscles (Tappel, 1960) contrasts with that described in the present paper for the *Carcinus* respiring particles. This difference only adds to the confusion regarding the role of cytochrome b in the respiratory chain (Slater, 1958).

The absolute concentrations of cytochrome a_3 in the hepatopancreas particles agree well with the value quoted for rat heart sarcosomes (see Table 1). This latter value has been recalculated from the data given by Chance & Baltscheffsky (1958) using the methods described by Chance (1954). There are no major differences in the turnover numbers for cytochrome a_3 in the various preparations listed in Table 1. Thus cytochromes a, a_3, b, c and c_1, flavoproteins and pyridine nucleotides appear to satisfy Slater's first criterion.

TABLE 1—A COMPARISON OF THE RESPIRATORY CHAIN OF THE HEPATOPANCREAS RESPIRING PARTICLES WITH THOSE OF MAMMALIAN RESPIRING PARTICLES AND INTACT INVERTEBRATE MUSCLE

The data included in this table are derived from formula and facts presented by Chance (1952, 1954), Chance & Williams (1955a), Chance & Baltscheffsky (1958) and Tappel (1960)

Source of respiring particles	Component						Substrate	q_{O_2} (μl/hr/mg protein)	a_3 content (μmoles/g protein)	Turnover number of cytochrome a_3
	a_3	a	b	$(c+c_1)$	Flavo-protein	Pyridine nucleotide				
Carcinus maenas hepato-pancreas	1·0	1·3	0·9	1·1	2·9	18·7	Succinate	30–40	0·19±0·02 (15 determinations)	12
Rat heart sarcosomes	1·0	0·9	0·9	1·2	1·0	4·0	α-Oxo-glutarate	75	0·20*	19
Rat liver mitochon-dria	1·0	0·8	0·4	1·1	2·0	30·0	DL-β-Hydroxy-butyrate	—	—	5
Callinectes sapidus, intact muscle	1·0	0·9	0·2	0·6	2·2	—	—	—	—	—
Homarus americanus intact muscle	1·0	0·9	0·1	0·6	1·3	—	—	—	—	—

* This figure has been recalculated from the data presented in Table 2 of Chance & Baltscheffsky (1958). The figure originally quoted was 0·7 μmoles cytochrome a_3/g protein.

It should be emphasized that the data concerning the hepatopancreas respiring particles (listed in Table 1) are calculated using expressions and wavelengths chosen by Chance and his co-workers for enzyme and respiratory chain preparations of mammalian origin. It remains to be shown whether the application of these expressions to these less-characterized non-mammalian respiratory chains is justified. The importance of this reservation is emphasized by the report that the muscles of some marine invertebrates contain two types of cytochrome *b* and an atypical cytochrome *a* (Tappel 1960) and also by the "non-mammalian" pattern of light absorption between 590–615 mμ by the *Carcinus* respiring particles (see Fig. 3). However, Chance (1952) and others, e.g. Tappel (1960), have assumed that this treatment may be applied to non-mammalian systems such as flight muscle

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mitochondria, pea seedlings and invertebrate muscle tissues. Holton (1955) has expressed doubts about the absolute validity of data similar to those presented in Table 1, and his objections have not yet been refuted. But his criticism does not detract from the comparative value of the data.

While no attempt has been made to satisfy the second criterion, the present results partially satisfy the third criterion. Thus, all the postulated members of the respiratory chain in the hepatopancreas respiring particles undergo oxidation and reduction during operation of the system. This is shown by the success of the reversal technique at all the wavelengths studied. Assuming that cytochrome a_3 is the major oxidase present in these particles, the velocity of the change from the aerobic to the anaerobic steady state at 445 mμ can be used as a guide to indicate whether a postulated component undergoes reduction with the requisite speed. On this basis, the pyridine nucleotides are clearly rejected from the respiratory chain associated with the oxidation of succinate. The experimental evidence for this rejection is illustrated in Fig. 6. This shows that whereas the reduction of the pigment at 445 mμ took less than 1 min, the reduction of the pyridine nucleotide took 6 min. However, at all other wavelengths the speed of reduction was similar to that at 445 mμ. Hence cytochromes *a*, a_3, *b*, *c* and c_1 and flavoproteins satisfy the third criterion.

In view of the uncertainty about the position of cytochrome *b* in the mammalian non-phosphorylating respiratory chain preparations (Slater, 1958), it is worth noting that as the preparation became reduced, the velocity of the changes in extinction in those regions of the difference spectrum of the hepatopancreas respiring particles where cytochrome *b* might be expected to contribute was as rapid as in other parts of the spectrum. This rapid reduction of cytochrome *b* is similar to that noted in other phosphorylation chains (Chance, 1954; Chance & Williams, 1955b, c; Chance & Baltscheffsky, 1958).

The diphasic kinetics at 445 mμ appears to be a new type of evidence for the existence of at least two pigments with absorption bands in this region; the pigments approaching complete reduction at different rates. These pigments could well be cytochrome *a* and a_3, both of which are known to absorb light strongly in this region (Yonetani, 1960). It is interesting to note that Lundegårdh (1957) suggests that cytochrome a_3 is reduced more rapidly than cytochrome *a* in intact yeast cells.

The main conclusion that may be drawn from the results presented here is that *Carcinus maenas* contains respiratory pigments which undergo alternate oxidation and reduction. These pigments are spectroscopically very similar to those which form part of the mammalian respiratory chain, namely, cytochromes *a*, a_3, *b*, *c*, c_1, flavoproteins and pyridine nucleotide-linked proteins. The original report of the presence of cytochromes in crustacean tissue was by Ball & Meyerhoff (1940), who, by direct spectroscopic observations, showed that cytochromes *a*, *b* and *c* were present in the heart muscle of *Homarus*. Tappel (1960) has investigated the difference spectra of muscle from three species of crustacea. His results indicate that the muscle of *Callinectes sapidus*, *Homarus americanus* and *Pandulus* sp. also contain

cytochromes *a*, a_3, *b*, *c*, c_1 and flavoproteins. But there are sufficient variations in these difference spectra to prevent any generalization on the respiratory chain of the crustacea.

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STUDIES ON *MYXINE GLUTINOSA*—I. THE CHEMICAL COMPOSITION OF THE TISSUES

D. BELLAMY and I. CHESTER JONES

Department of Zoology, University of Sheffield

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Abstract—(1) Determinations were made of the concentrations of the major low molecular weight constituents in serum, muscle and liver of *Myxine glutinosa* L. (the Atlantic hagfish).

(2) The intracellular concentrations of the above substances have been calculated using the inulin space as a measure of the extra-cellular fluid compartment of the tissues.

(3) The concentrations of potassium, sodium and chloride in the intracellular water were 140–200, 110–150 and 100–120 mM/kg respectively. For potassium the ratio of the intracellular to extracellular concentration was 12 : 1. The corresponding ratio for sodium was 1 : 4·5.

(4) From the composition of the various tissues it is likely that serum and cells had the same osmotic pressure as sea water. Serum contained mainly inorganic ions and the intracellular fluid contained approximately equal quantities of inorganic and organic constituents.

(5) The major intracellular organic compounds were amino acids (20–70 mM/kg H_2O), trimethylamine oxide (211–230 mM/kg H_2O) and an unknown acidic substance (about 200 mM/kg H_2O).

INTRODUCTION

THE normal serum of the hagfishes *Myxine glutinosa* L. and *Polistotrema stouti* has almost the same ionic composition as that of the surrounding seawater (Robertson, 1954; McFarland & Munz, 1958). Analysis of muscle has shown an inorganic ion content of only 41 per cent of that of seawater and the nature of the deficit is a matter of conjecture (Robertson, 1960). In a series of investigations on the adrenal cortex of vertebrates, it was shown that the blood of *P. stouti* contained significant quantities of cortisol and corticosterone of the same order as those in many representatives of the Gnathostomata (Chester Jones and Phillips, 1960). In seeking a functional basis for the presence of these steroid hormones, the findings of Robertson (1954, 1960) were confirmed and extended and this paper gives the results of measurements of extracellular space, and the major intracellular ions in muscle and liver of *M. glutinosa*.

MATERIALS AND METHODS

Animals. The hagfish were obtained from Kristinebergs Zoologiska Station, Sweden, or from St. Andrew's Marine Station, New Brunswick, Canada, sent by air and kept in the Departmental Aquarium at 10°C in seawater obtained from the Channel or the North Sea.

Extraction of tissues. Tissue samples were dipped rapidly into about 500 ml distilled water and then drawn across a dry glass surface to remove surplus water. The samples were weighed on a torsion balance, and placed in test tubes with 2 ml 0·1 N HNO_3 per 200 mg wet wt. After 24 hr at room temperature, the nitric acid solution was decanted from the solid tissue and used for the estimation of potassium, sodium, chloride, calcium, magnesium and phosphate. In the procedure for the extraction of free amino acids and trimethylamine oxide, nitric acid was replaced by 5 per cent trichloracetic acid (TCA).

Dry weights. Other tissue samples were washed, drained and weighed as above and then dried for about 18 hr at 110°C.

Blood serum. The body cavity was opened and blood obtained from the heart with a 1 ml hypodermic syringe. After a period of 30 min at room temperature the blood was centrifuged at 1000 g for 10 min to separate the clear straw-coloured serum. The serum was discarded if tinged with red.

Serum was diluted 1/2000 with distilled water for the determination of sodium and potassium. For other ions one-tenth of the volume of 50 per cent TCA was added to the serum, the mixture centrifuged at 2000 g for 10 min and the supernatant fluid used.

Estimation of sodium, potassium and chloride. These ions were estimated as described previously (Bellamy, 1961).

Estimation of calcium and magnesium. The sum of calcium and magnesium was determined by titration with disodium ethylene diaminetetra-acetate (EDTA) using Eriochrome Black T. as the indicator (Griswold & Pace, 1956). Calcium was estimated similarly by titration with EDTA using calcein as the indicator (Bett & Fraser, 1958). Magnesium was determined by difference.

Total phosphate. Total phosphate was determined by the method of Berenblum & Chain (1938) as modified by Bartley (1953).

Free amino acids. Free amino acids in 5 per cent trichloracetic acid extracts were determined by the method of Krebs & Bellamy (1960).

Trimethylamine oxide. Trimethylamine oxide was chromatographed and identified by the methods of Levine & Chargaff (1951), Bregoff *et al.* (1953) and Baker & Chaykin (1960). Trimethylamine oxide was the main compound visible on the chromatograms. Smaller quantities of other quaternary ammonium compounds appeared to be present. Quantitative determinations of trimethylamine oxide were carried out by the method of Kermack *et al.* (1955).

Non-protein nitrogen. About 400 mg wet wt. of tissue was broken up with a glass rod in a test tube with 2 ml distilled water. Then 0·2 ml of 10 per cent sodium tungstate was added, followed by 0·2 ml of 0·5 N H_2SO_4. After 18 hr at room temperature the mixture was centrifuged at maximum speed in an MSE bench centrifuge and an aliquot (1·0 ml) of the supernatant solution was added to 5 ml of a 1 : 1 mixture of conc. HNO_3 and conc. H_2SO_4 (containing 1 per cent SeO_2) in a 50 ml Kjeldahl flask. The contents of the flask were heated until colourless and made up to 100 ml with distilled water. Ammonia in the resultant

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solution was determined by adding Nessler's reagent (1·0 ml to 3·0 ml solution) and reading the optical density at 475 mμ.

Inulin space. The inulin space of muscle and liver was determined by a method following Tasker *et al.* (1960). Whole "tongue" muscles (M. longitudinalis linguae; Cole, 1907) were placed in 25 ml conical flasks with 10 ml of a solution containing 542 mM NaCl; 16 mM $MgCl_2$; 7 mM K_2SO_4; 6 mM Na_2HPO_4; 3 mM $CaCl_2$ and 10 g inulin per 1000 g H_2O. The flasks (open to the atmosphere) were shaken in a Dubnoff metabolic incubator at 15°C. After incubation, the muscles were divided into two. One piece was transferred to a conical flask containing 100 ml of inulin-free saline and the inulin in the muscle was allowed to reach diffusion equilibrium (18 hr at 5°C). The inulin content of the saline was then determined by the method of Kulka (1956). The dry weight and ion content were obtained from the second piece of muscle.

Slices of liver about 0·5 mm thick were cut with a razor blade and treated by the same procedure as for whole tongue muscle. Tongue muscle was chosen because it could be removed from the body as a whole muscle and therefore was suitable for determination of the extracellular space by incubation in an inulin medium. A second procedure was used with parietal muscle in order to avoid overestimating the extracellular space due to the penetration of inulin through the cut ends of the muscle fibres. A 3 per cent solution of inulin in sea water (1 ml per 50 g body wt.) was injected into the peripheral subcutaneous sinus and samples of blood and parietal muscle were removed 4 hr later. The inulin content of the muscle was obtained as above. Blood samples were allowed to clot at room temperature for 30 min, the serum decanted and the inulin concentration determined after diluting 1/100 with deionized water. Extracts of control tissue containing no inulin gave negligible colour development.

RESULTS

Constituents of liver and muscle

Sodium, potassium and chloride were found to be the major inorganic ions in the liver and muscle (Table 1). Sodium and chloride were present in equal amounts in the liver. Muscle contained about 17 per cent more sodium than chloride. The concentration of potassium was about the same in both tissues and was considerably lower than that of sodium. These ions, in liver for example, together with phosphate, magnesium and calcium gave a total of 691 mM inorganic ions per kg muscle water. This is about 40 per cent lower than the osmolarity of the serum and raises the possibility of the tissue cells being hypotonic to blood. The high concentration of nitrogenous compounds, comprising amino acids and trimethylamine oxide, when added to that of the inorganic constituents, gave a total solute concentration of about 800 mosmoles/kg tissue water. The tissue osmotic pressure still did not balance that of the serum, made up for the most part by inorganic constituents. Since the amino acids and trimethylamine oxide can exist in the cell as positive ions, there was a possibility that these compounds were balanced by an organic acid, a situation occurring, for example,

in squid nerve (Deffner & Hafter, 1960). With this possibility in mind, extracts of muscle and liver were fractionated into basic, neutral and acidic fractions with ion exchange resins (Koechlin, 1955). A substance, which was not inorganic chloride, phosphate or sulphate was found in the acidic fraction from both tissues. The compound has not been identified as yet but it behaved as a weak monobasic acid, equivalent to about 120 mM per kg tissue H_2O. Adding this amount of acid then, the total osmolarity of the tissue balances within reasonable limits (85 per cent) that of the serum.

TABLE 1—CONCENTRATION OF SUBSTANCES IN THE TISSUE WATER OF LIVER AND "TONGUE" MUSCLE

Tissues were extracted and estimations carried out as described in the text. The water content was 695 ± 55 mg/g wet wt. and 757 ± 11 mg/g wet wt. for liver and muscle respectively. Ten experiments were made in each case

Substance	Concentration in tissue water (mM/kg H_2O)	
	Liver	"Tongue" muscle
Sodium	248·0 ± 11·7	207·0 ± 13·2
Potassium	135·0 ± 11·3	146·0 ± 5·7
Magnesium	12·7 ± 3·6	17·5 ± 0·63
Calcium	2·48 ± 0·24	2·96 ± 0·57
Chloride	235·0 ± 9·8	172·0 ± 9·21
Total phosphate	58·7 ± 2·0	75·3 ± 1·4
Non-protein nitrogen	189·0 ± 8·3	274·0 ± 7·7
Free amino acids	13·6 ± 0·75	61·3 ± 0·61
Trimethylamine oxide	159·0 ± 8·4	182·0 ± 6·1

Inulin space and intracellular ions

Table 1 gives concentrations expressed in terms of total tissue water. Determinations of the extracellular space are required in order to know the distribution of ions across the cell membrane. Tasker *et al.* (1960) showed that the penetration of inulin into the tissues of poikilothermic vertebrates gave reliable values for the extracellular space. This method, therefore, was chosen for the muscle and liver of *Myxine*.

When tissues were incubated with inulin in a medium which closely resembled the serum of *Myxine* in ion content, a maximum penetration of inulin occurred after 2·5 hr (Table 2). No change in either the wet weight of the tissue or the sodium and potassium content was detected during this time. The amount of inulin found in muscle corresponded to an average penetration of 13·7 per cent of tissue water; in liver to 32 per cent. Using these values, the intracellular concentration of the major constituents of liver and muscle were calculated and given in Table 3. The calculations required the assumption that the extracellular fluid volume (inulin space) contained ions at the same concentration as in the serum.

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The results show that not only was potassium, as would be expected, present intracellularly in high concentration but so also were sodium and chloride. This finding is of particular interest as it is not usual in higher vertebrates. The potassium distribution ratio between the intracellular and extracellular fluid was 13–14 : 1 corresponding to a membrane potential of about 65 mV. In fact the

TABLE 2—INULIN SPACE OF ISOLATED LIVER AND TONGUE MUSCLE

Isolated whole muscles were incubated for various times as described in the text in 10 ml saline containing 542 mM NaCl; 16 mM $MgCl_2$; 7 mM K_2SO_4; 6 mM Na_2HPO_4; 3 mM $CaCl_2$ and 10 g inulin per 1000 g H_2O. Temp. 15°C. Several liver slices were incubated in the same way and individual slices removed at the stated times. The amount of inulin which had penetrated the tissues was determined as described in the text

Time of incubation (hr)	Inulin space (Amount of inulin as ml of a 1% soln.) per 100 ml tissue H_2O)	
	Liver	"Tongue" muscle"
1·0	18·5 ± 2·4	8·13 ± 0·59
2·0	28·8 ± 3·2	11·8 ± 1·0
2·5	32·0 ± 2·6	13·7 ± 1·1
3·0	31·1 ± 3·7	12·6 ± 0·8

TABLE 3—CONCENTRATION OF SUBSTANCES IN THE TISSUE FLUIDS OF LIVER AND MUSCLE

The concentration of ions in the intracellular water was calculated from the ion content of the whole tissue assuming that the inulin space contained ions at the same concentration as in serum

Substance	Ion content (mM/kg H_2O)			
	Sea water (1 expt.)	Serum (7 expts.)	Intracellular water	
			Liver (7 expts.)	"Tongue" muscle (6 expts.)
Potassium	12·2	11·1 ± 0·5	161·0	144·0
Sodium	470·0	549·0 ± 4·0	110·0	132·0
Chloride	550·0	563·0 ± 16·3	122·0	107·0
Calcium	8·4	5·1 ± 1·2	1·3	2·62
Magnesium	49·7	18·9 ± 1·5	9·8	17·5
Phosphate	—	5·0 ± 0·3	84·2	86·4
Trimethylamine oxide*	—	—	234·0	211·0
Amino acids*	—	—	20·0	71·0
Unidentified N compounds	—	—	25·0	35·7
Acidic fraction	—	—	202·0	183·0
Total osmolarity of measured ions	1067	1152	969·0	990·0

* None detected, i.e. less than 1·0 mM trimethylamine oxide and amino acids in serum.

membrane potential of the fibres in the "tongue" muscle was found to be 72 mV at 20°C (experiment kindly carried out by Dr. P. Andersen in Oslo). From the latter figure one would expect a ratio of 17 : 1 for the concentration of intracellular to extracellular potassium if this ion was distributed passively. The agreement between the two results is thus very close.

One half of the intracellular osmotic pressure is apparently due to the presence of organic molecules to which the cell membrane is impermeable. The question arises as to the source of these organic compounds, particularly in view of evidence that the trimethylamine oxide in teleost muscle may be derived from that in the food (Benoit & Norris, 1945). Because of the importance of trimethylamine oxide in maintaining osmotic equilibrium between blood and tissues, it is likely that this substance is synthesized endogenously. This is indicated by unpublished experiments which show that trimethylamine, but not methylamine, was more effective than glucose in increasing the oxygen uptake of homogenates of *Myxine* liver (cf. Baker & Chaykin, 1960).

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Osmotic equivalence

The total osmolarity of the external environment (1067 mosmoles/kg H_2O) was almost the same as that of serum (1151 mosmoles/kg H_2O). Although the concentration of all possible ions was not measured, the results so far obtained support the conclusions of Robertson (1954) that the serum of *M. glutinosa* is in osmotic equilibrium with sea water. The intracellular ions (990 mosmoles/kg water) amounted to about 86 per cent of the total measured in sea water, the percentage deficit being only slightly larger than the standard error of most of the individual measurements. Since tests were not made for neutral intracellular compounds (e.g. low molecular weight carbohydrates) which are known to occur in the cells of marine invertebrates (Deffner & Hafter, 1960), it is very probable that the cells and blood are in osmotic balance.

DISCUSSION

Sodium content of cells

The Myxinoidea, of which *M. glutinosa* is a representative, occupy a unique position in the vertebrate series, not least in that the serum has almost the same composition as sea water. In the cell the potassium content is not exceptional, falling within the range for a wide variety of animals. However, the intracellular concentration of sodium, namely 110–132 mM/kg H_2O, is unusually high for vertebrates and also for some invertebrates, where the normal range is 16–50 mM/kg H_2O (e.g. Conway & Hingerty, 1946; Shaw, 1955a).

The reason for the high intracellular sodium and chloride concentrations in *Myxine* is not clear. It is true that in some vertebrate tissues large amounts of intracellular sodium occur [e.g. in the heart (Davies *et al.*, 1952) and in some red cells (Krogh, 1946; Bernstein, 1954)]. However, it may be that in the hagfish the concentration of intracellular sodium represents an equilibrium between an unusually high passive influx (due to a high extracellular concentration of sodium)

and an active efflux of this ion. On these grounds, the situation in *Myxine* would then be no different in principle from that in the Eutheria. In the latter, a sodium concentration of 150 mM/kg serum H_2O results in a concentration of 16 mM/kg intracellular H_2O, a ratio of about 9 : 1 (Conway & Hingerty, 1946), whilst in *Myxine* with 550 mM sodium/kg serum H_2O, the ratio is about 5 : 1.

With particular reference to parietal muscle another possibility exists, namely that the tissues of *Myxine* are not exactly of the vertebrate type. The parietal muscle has not been examined by modern techniques but it appears to be composed of both red and white fibres and gives a histological picture not unlike that of M. pectoralis major of the pigeon (cf. Cole, 1907; George & Naik, 1959). In the present study, parietal muscle was found to contain 202 mM of potassium and 152 mM of sodium per kg intracellular H_2O (inulin space 15·0 per cent). These values represent the ion content of the mixed fibres. If the fibres differed in function it is possible that they would also differ chemically; some fibres might contain more sodium than others. It is interesting, therefore, that Andersen (personal communication) recorded two different resting potentials for the parietal muscle fibres of *M. glutinosa*. Type 1 fibres (white fibres of Cole, 1907) had a membrane potential of 78 mV. The potassium concentration in these fibres would be expected to be at least 200 mM/kg H_2O if sodium was excluded. Type 2 fibres (red fibres of Cole, 1907) had a membrane potential of 50 mV corresponding to an expected intracellular potassium concentration of about 70 mM/kg H_2O.

The inorganic analysis of the mixed parietal muscle fibres together with Andersen's results suggest that the parietal muscle is composed of some fibres with a ratio of sodium to potassium greater than unity and others in which the ratio is reversed. This situation appears to be similar in some respects to that in *Pecten* where the catch fibres (slow acting) are high in sodium and the posterior fibres (fast acting) have a low sodium content (Potts, 1958). However, in *Pecten* the sodium content is lower than that of potassium in both types of fibre.

Yonge (1936) regarded the slow muscle of molluscs, subsequently shown by Potts (1958) to be high in sodium, as a primitive tissue. This might imply that the first living cells contained more sodium than potassium. It may be of significance in this respect that the concentration of intracellular sodium in mammals is higher during the initial stages of foetal growth than at birth (Dickerson & Widdowson, 1960). Indeed, Klein (1960) considered that the early cells of the chick embryo with a sodium to potassium ratio greater than unity might resemble those at a primary stage in the evolution of living organisms. Such an idea is not out of keeping with Conway's (1943) calculations for the composition of the mid-Precambrian sea, namely 203 mM sodium and 72 mM potassium. This ionic relationship might have led in the first place to the formation of cells with the composition postulated for Andersen's Type 2 fibres.

Trimethylamine oxide and organic acids

Not only do inorganic ions contribute to the osmotic pressure of the myxinoid cell, but so also do significant amounts of trimethylamine oxide and organic acids.

Myxine resembles elasmobranchs and cephalopods in the quantity of trimethylamine oxide in the tissues. Trimethylamine oxide has also been found in other marine molluscs and in crustacea (Norris & Benoit, 1945). In these organisms, as in *Myxine*, organic acids, especially amino acids, appear to make a substantial contribution to the intracellular osmotic pressure (Shaw, 1955b; Potts, 1958). The presence of a high concentration of an organic acid which is not an amino acid, although an unusual feature, is also a characteristic of cephalopods (Koechlin, 1955; Deffner & Hafter, 1960).

All these findings indicate that it is only in their potassium content that the cells of *M. glutinosa* resemble those of the higher vertebrates. In other respects the myxinoid cell has much in common with the cells of marine invertebrates, particularly those of the class Mollusca. Either the latter similarities to animals with an unequivocally marine origin are coincidental or the ancestors of the Agnatha might themselves be regarded as primarily sea dwellers (Robertson, 1957).

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CARBOHYDRATE METABOLISM IN THE TWO-SPOTTED SPIDER MITE, *TETRANYCHUS TELARIUS* L.*—I. HEXOSE MONOPHOSPHATE CYCLE

K. N. MEHROTRA

Department of Entomology, Cornell University, Ithaca, N.Y.

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Abstract—(1) Evidence demonstrating the occurrence of the hexose monophosphate oxidative pathway in the two-spotted spider mite *Tetranychus telarius* (L.) has been obtained.

(2) It has been shown that the two-spotted spider mite contains active glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, phosphopentose isomerase, phosphoketopentose epimerase, transketolase and transaldolase.

(3) This oxidative pathway cycle consists of the following main steps: (a) two-step oxidation of glucose-6-phosphate to pentose phosphate and CO_2; (b) the conversion of pentose phosphate to sedoheptulose phosphate and triosephosphate; and (c) the conversion of sedoheptulose phosphate to hexose phosphate.

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INTRODUCTION

CARBOHYDRATE metabolism as known in most organisms may be outlined within the limits of three major pathways: glycolysis, the tricarboxylic acid cycle, and the hexose monophosphate cycle (pentose cycle). Although information of this type is available for several insect species (see reviews by Chefurka, 1958b; and Sacktor, 1958) and other vertebrates and invertebrates (Bueding and Farber, 1960; Axelrod, 1960), detailed studies on the metabolism of carbohydrates in the Acarina have not been reported. Moreover, it seems essential that before any real understanding of the physiology of an organism can be attained, basic studies must establish whether the initial processes of food utilization are similar to or differ from comparable processes in other organisms.

The present communication is concerned with the presentation of individual enzymatic reaction patterns of the hexose monophosphate cycle in the two-spotted mite, *Tetranychus telarius* (L.).

MATERIALS AND METHODS

Reagents. 6-phosphogluconate (6PG), glucose-6-phosphate (G-6-P), ribose-5-phosphate (R-5-P), triphosphopyridine nucleotide (TPN^+) and reduced diphosphopyridine nucleotide (DPNH), purified lactic dehydrogenase, acid phosphatase and 6-phosphogluconic dehydrogenase were purchased from Sigma Chemical

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Company, St. Louis, Mo. A quantity of sedoheptulosan was kindly provided by Dr. W. Chefurka, Pesticide Research Institute, London, Ontario. Other chemicals used were of reagent grade of highest purity.

Mites. Adult mites were obtained from a laboratory culture maintained on Fordhook U.S. 242 lima beans grown at 80 ± 3°F temperature, 60 per cent humidity and under 500 ft-c constant illumination. Homogenates of the adult mites were prepared by grinding the mites in an ice-cold all-glass Potter-Elvehjem homogenizer. The homogenate was lightly centrifuged to remove larger pieces of cuticle, and the supernatant kept in crushed ice until used.

Assay methods. Glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase were assayed spectrophotometrically according to Glock and McLean (1954). Transketolase was assayed according to Horecker *et al.* (1953). All spectrophotometric measurements were made at room temperature with a Beckman Model DU Spectrophotometer.

The pentoses were determined according to Mejbaum (1939) with 40 min heating using the extinction coefficient given by Newburg and Cheldelin (1955). Whenever necessary the pentose values were corrected for interference by heptoses. The heptoses and hexoses were assayed after 18 hours with the sulphuric acid–cysteine method (Dische *et al.*, 1949) using the molar extinction coefficient given by Newburg and Cheldelin (1955). Ketopentoses were determined by the cysteine–carbazol method (Dische & Borenfreund, 1951).

Paper chromatography

The ascending method of Williams and Kirby (1948) was followed for the identification of pentoses. Chromatograms were made on Whatman No. 1 filter paper with (a) 77% acetone and (b) acetone–water (10 : 3) saturated with boric acid as the solvent. The latter solvent system permitted a good separation of aldopentoses and ketopentoses. The pentoses were located on the chromatograms by spraying with aniline hydrogen phthalate (Partridge, 1949) and heating for 5 min.

RESULTS

Glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase

The following reactions are catalyzed by the enzymes glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase.

$$\text{G-6-P} + \text{TPN}^{+} \xrightarrow[\text{dehydrogenase}]{\text{G-6-P}} \text{6PG} + \text{TPNH} \qquad (1)$$

$$\text{6PG} + \text{TPN}^{+} \xrightarrow[\text{dehydrogenase}]{\text{6PG}} \text{Ribulose 5-Phosphate} + \text{CO}_2 + \text{TPNH} \qquad (2)$$

The data presented in Fig. 1 indicate that both these dehydrogenases were active in the presence of their respective substrates. When pyruvate and lactic

dehydrogenase, which is capable of reacting with TPNH (Mehler *et al.*, 1948), were added to this system the pyruvate was rapidly reduced with oxidation of TPNH according to the reaction.

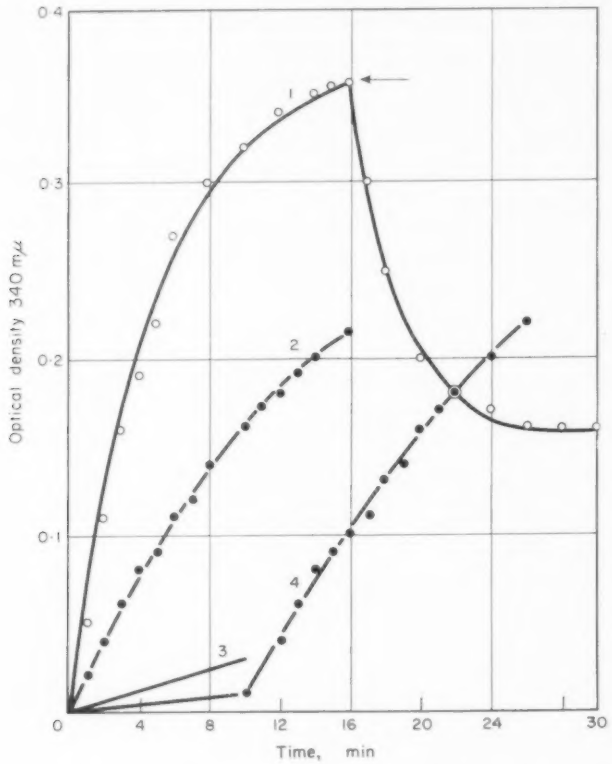

FIG. 1. Glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase activities of the two-spotted spider mite. The reaction cuvette contained 20 μ mole of $MgCl_2$, 0·2 μ mole of TPN^+, 150 μ mole of glycylglycine buffer pH 7·6, 0·2 ml of mite homogenate (25 mg/ml) and in: (1) 5 μ mole of glucose-6-phosphate and 5 μ mole of 6-phosphogluconate. At the point of arrow 10 μ mole of sodium pyruvate and 2 units of lactic dehydrogenase were added (1 unit of lactic dehydrogenase equal to the enzyme which produced 0·01 μ mole lactate per minute). (2) 5 μ mole of 6-phosphogluconate. (3) No substrate. (4) 2·5 μ mole of glucose-6-phosphate and 2·5 μ mole 6-phosphogluconate. Initially it contained 0·2 μ mole of DPN but no TPN; 0·2 μ mole TPN was added at 10 minutes (mark of arrow). Total volume 1·2 ml.

$$\text{Pyruvate} + \text{TPNH} \xrightarrow[\text{dehydrogenase}]{\text{lactic}} \text{Lactate} + \text{TPN}^+ \qquad (3)$$

Both these enzymes seem to be TPN specific, because no appreciable reduction of DPN^+ was observed with an enzyme preparation which reduced TPN^+ at the rate of 0·05 optical density units/min. It is noteworthy that these mites contain

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approximately equal amounts of G-6-P dehydrogenase and 6PG dehydrogenase rather than a preponderance of G-6-P dehydrogenase as in house flies (Chefurka, 1955) or of 6PG dehydrogenase as in the fat body of the cockroach (Young, 1958).

Stoichiometry and the identity of reaction products of 6PG *oxidation*

The stoichiometry and the nature of the reaction products of 6PG oxidation were studied by coupling this oxidation to the reduction of pyruvate in the presence of lactic dehydrogenase. In this way the use of stoichiometric amounts of TPN^+

TABLE 1—STOICHIOMETRY OF 6-PHOSPHOGLUCONATE OXIDATION

Expt. No.	Duration (minutes)	Phosphogluconate (μ mole/ml)		Pentose (μ mole/ml)
1	30	Initial	5·0	2·0
		Final	1·5	5·6
			−3·5	+3·6
2	30	Initial	15·0	2·3
			11·0	6·2
			−4·0	+3·9

The reaction mixture contained the following μ mole/ml in addition to initial quantities shown in Table, $MgCl_2$, 5·0; glycylglycine buffer (pH 7·6), 20; TPN^+, 0·2; sodium pyruvate, 10; lactic dehydrogenase, 0·1 mg protein and 1·0 ml homogenate (100 mg/ml). Total volume 5·0 ml.

was avoided. The reduced TPN^+ produced in the oxidation of 6PG interacts with pyruvate which in turn is reduced to lactic acid. The reoxidized TPN^+ can then react again with another molecule of 6PG. This sequence of events continues until the substrates are utilized.

The data shown in Table I indicate that disappearance of 6PG coincides with the appearance of an equivalent amount of pentoses as measured by the orcinol test.

The identity of the pentoses appearing in the reaction mixture was established by paper chromatography. Early attempts to isolate the pentoses by using crude homogenate as enzyme source and a longer period of incubation were unsuccessful, presumably due to the interference of transketolase and transaldolase which converted the pentoses immediately into heptuloses and hexose. Therefore, the enzyme was partially purified so as to eliminate the enzymes transketolase and transaldolase. This was accomplished by heating the homogenate to 60°C for five minutes. By this heat treatment the activities of transketolase and transaldolase were completely destroyed without an appreciable reduction in the activity of 6-phosphogluconic dehydrogenase, phosphopentose isomerase and phosphopentose epimerase; only 20 per cent reduction in the 6-phosphogluconic dehydrogenase activity was observed. The reaction mixture was similar to one given in Table 1 except that heated homogenate (as described previously) was used. The reaction

was stopped by boiling for 5 min, the pH was adjusted to 5·0. The sugar phosphates were dephosphorylated by the addition of 5 ml of 0·2 M pH 5·0 acetate buffer, 2 ml of 0·1 M of $MgCl_2$ and 25 mg of acid phosphatase. At the completion of

TABLE 2—PAPER CHROMATOGRAPHY OF PENTOSES

Solvent	Reaction product R_f	Ribose R_f	Xylulose* R_f
77% acetone	0·65, 0·79	0·63	0·76
Acetone–water (10 : 3) saturated with boric acid	0·56, 0·88	0·86	0·57

* From the data of Chefurka (1958a).

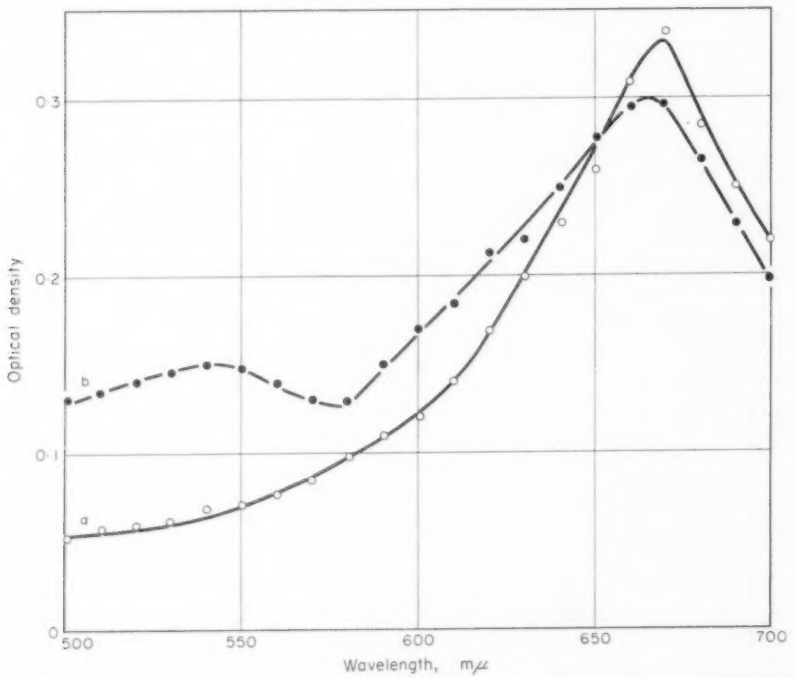

FIG. 2. Absorption spectra of the oxidation products of 6-phosphogluconate oxidation.
(a) orcinol test on the eluate of the component with R_f 0·88 in acetone–water–boric acid.
(b) orcinol test on the eluate of the component with R_f 0·56 in acetone–water–boric acid.

dephosphorylation the reaction was stopped by boiling for 5 min. This dephosphorylated mixture was deionized by passing through a mixed bed of Dowex-50 and IR-4B resin. The pentoses were eluted from the column and were recovered

in the first 200 ml. This was reduced to dryness under reduced pressure and the residue dissolved in 1 ml of deionized water. Small aliquots of these were subjected to paper chromatography. The data in Table 2 indicate the presence of two components with R_f's in both solvents identical with those of xylulose and ribose.

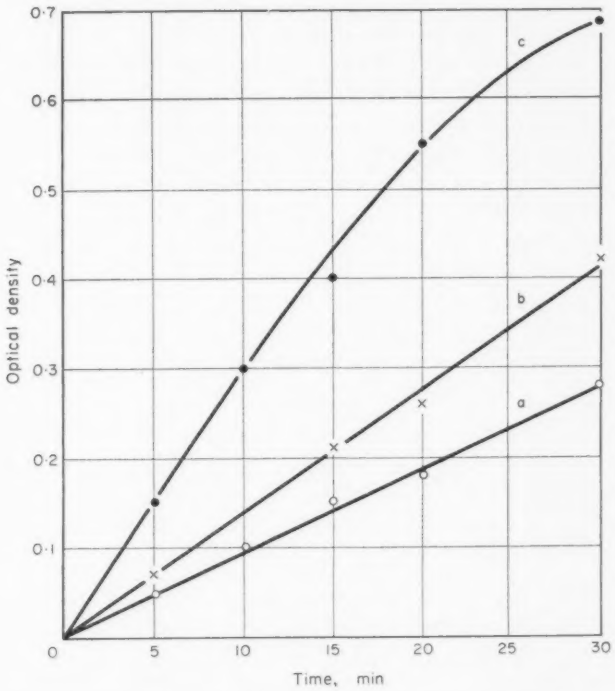

FIG. 3. Phosphopentose isomerase activity in the two-spotted spider mite. The reaction mixture contained the following (μ mole/ml) ribose-5-phosphate 4, tris buffer (pH 7·0) 5, and (a) 0·2 ml, (b) 0·3 ml and (c) 0·5 ml of the 'treated' mite homogenate (100 mg/l). Total volume 7·5 ml. The homogenate was heated to 65°C for 5 min before use.

A further confirmation of the identity of the components was obtained by eluting each of the fractions from paper and subjecting them to the orcinol test. Difficulty was encountered, at first, because of the presence of elutable substances in the paper, which reacted positively in the orcinol test. This was overcome by prewashing the paper with the solvents and by assaying a piece of paper equivalent to the size of the test spots as blank.

The data in Fig. 2 indicate that the component with the R_f 0·88 in acetone saturated with boric acid, when subjected to the orcinol test, exhibited only one strong maximum at 670 mμ in its absorption spectrum, and the ratio of the optical

densities at 540 and 670 mμ was 0·22. The other component ($R_f = 0·56$), on the other hand, exhibited an absorption spectrum with peaks at 670 mμ and 540 mμ. The ratio of the two peaks was 0·48. Both these characteristics are in agreement with those known for D-ribose and D-xylulose (Hochster, 1955; Seegmiller & Horecker, 1952; Chefurka, 1958a).

Phosphopentose isomerase

The presence of phosphopentose isomerase is evident from the fact that ribose appears as the product of 6PG oxidation. Moreover, the existence of phosphopentose isomerase in the two-spotted spider mite is also apparent from the assay of the enzyme transketolase according to Horecker & Smyrniotis (1953) (see below). However, a direct assay of this enzyme was made by the method of Axelrod (1955).

The rate of isomerization of the aldopentose to the ketopentose is seen in Fig. 3. The reaction product of isomerization of aldopentose was a ketopentose; this was confirmed by its ability to withstand bromine oxidation. The ketopentose was treated with an equal amount of 1·5% bromine water in the presence of 500 mg barium carbonate for 30 min. After removal of the bromine by aeration the solution was treated with cysteine–carbazol. No change in the ketopentose concentration was observed after this oxidation.

Phosphoketopentose epimerase

No direct attempt was made to demonstrate the presence of the enzyme phosphoketopentose epimerase, which catalyses the following reaction:

$$\text{ribulose-5-phosphate} \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\rightarrow \text{xylulose-5-phosphate} \qquad (4)$$

The presence of epimerase was shown indirectly, however, by following the formation of xylulose phosphate as a function of time during the conversion of aldopentose to ketopentose as described by Chefurka (1958a).

The data in Fig. 4 indicate that the amount of xylulose phosphate increased in proportion to the total ketopentose level with time of incubation. This suggests that ribulose phosphate was being converted to xylulose phosphate. If the difference in optical density between 120 min and 10 min is used as an approximate measure of xylulose, then very little, if any, xylulose phosphate formation occurred during the first minute of incubation. Some xylulose phosphate, however, appeared after 5 min and then increased thereafter with increasing time of incubation.

Conversion of pentose phosphate to hexose monophosphate

(1) *Enzymes.* Conversion of the pentose phosphate to hexose phosphate is known to take place in two steps. First, the pentose phosphate is converted to sedoheptulose-7-phosphate by transketolase (Horecker *et al.*, 1951, 1953). Second, sedoheptulose is converted to hexose monophosphate by transaldolase (Horecker & Smyrniotis, 1955).

The activity of transketolase was measured according to Horecker *et al.* (1953). The reaction proceeds as follows:

$$\text{ribulose-5-phosphate} \longleftrightarrow \text{triosephosphate} + \text{active glycoaldehyde} \quad (5)$$

$$\text{triosephosphate} + \text{DPNH} \longleftrightarrow \alpha\ \text{glycerophosphate} + \text{DPN}^{+} \quad (6)$$

the overall reaction being:

$$\text{ribulose-5-phosphate} + \text{DPNH} \longleftrightarrow \alpha\ \text{glycerophosphate} + \text{DPN}^{+} + \text{active glycoaldehyde} \quad (7)$$

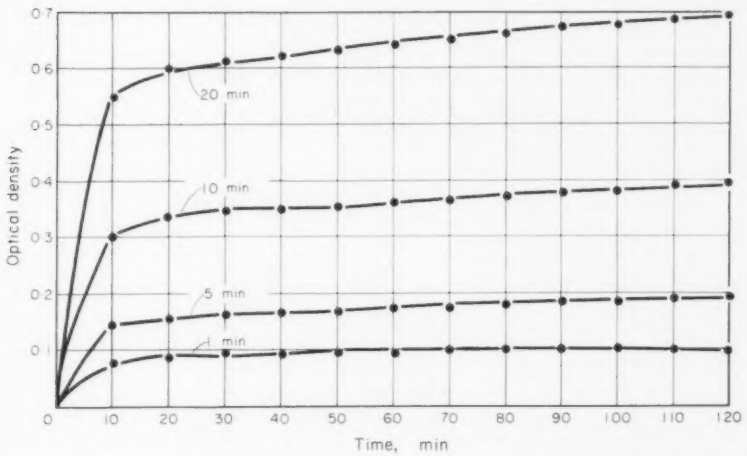

FIG. 4. The formation of xylulose phosphate as a function of time. The reaction mixture contained the following (μ mole): glycylglycine buffer (pH 7·5) 100, ribose-5-phosphate 20, and 3 ml of "treated" mite homogenate (100 mg/ml). Total volume 10 ml. At various periods of time 2·0 ml aliquots were withdrawn and added to 0·2 ml of 10 N HCl to stop the reaction. The pH was adjusted to 5·0 and each aliquot dephosphorylated with 15 mg acid phosphatase. The rate of color development was determined on 1 ml aliquots using the cysteine–carbazol test at 540 mμ.

Because these mites are known to possess an active α glycerophosphate dehydrogenase (Mehrotra, unpublished), DPNH should be oxidized if triosephosphate is a product of pentose breakdown. The data in Fig. 5 indicate an active transketolase as measured by rapid oxidation of DPNH in the presence of ribose-5-phosphate, which was probably first converted to ribulose-5-phosphate by pentose phosphate isomerase.

Attempts were not made to demonstrate the presence of transaldolase directly, which catalyzes the reaction:

$$\text{Sedoheptulose-7-phosphate} + \text{glyceraldehyde-3-phosphate} \dashrightarrow \text{fructose-6-phosphate} + \text{erythrose-4-phosphate} \quad (8)$$

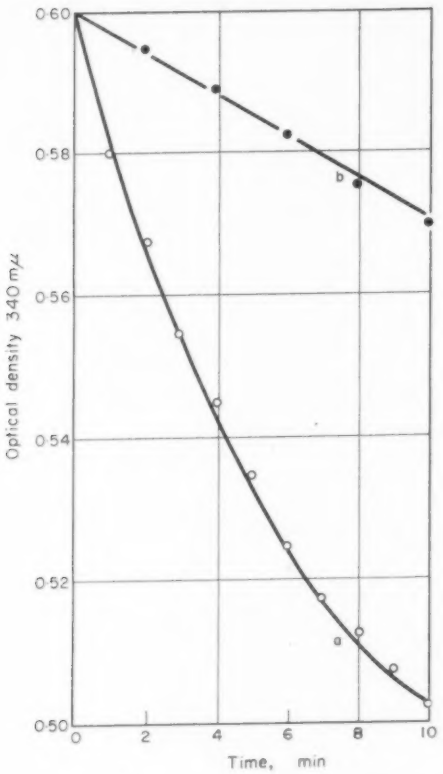

FIG. 5. Transketolase activity of the two-spotted mite. The reaction cuvette (a) contained 15 μ mole $MgCl_2$, 5·0 μ mole cysteine, 0·2 μ mole DPNH, 9·0 μ mole ribose-5-phosphate, 150 μ mole glycylglycine buffer pH 7·5, 1 ml homogenate (50 mg/ml). Blank cuvette (b) contained no substrate. Total volume 3·0 ml.

TABLE 3—STOICHIOMETRY OF RIBOSE-5-PHOSPHATE BREAKDOWN

Time (hr)	Ribose consumed (μ mole)	Heptulose formed (μ mole)	Hexose formed (μ mole)	Hexose + heptulose (μ mole)
0·5	15	6·0	0·2	6·2
1·0	17	6·9	1·9	8·8
2·0	21	3·0	6·5	9·5
3·0	22	2·4	6·0	8·4

The reaction tube contained 1×10^{-3} M ribose-5-phosphate, 0·04 M glycylglycine buffer, pH 7·6; $3{\cdot}0 \times 10^{-2}$ M NaF; 5·0 ml homogenate containing 300 mg of mites and water to make a volume of 30 ml. Determinations were made on aliquots withdrawn at different time intervals and deproteinized with trichloroacetic acid. In the control incubation active enzyme was replaced by boiled enzyme.

The presence of transaldolase is obvious, however, from the fact that the hexose phosphate along with the heptulose phosphate appeared as a product of pentose breakdown (Fig. 6). Moreover, the fact that sedoheptulose appeared before

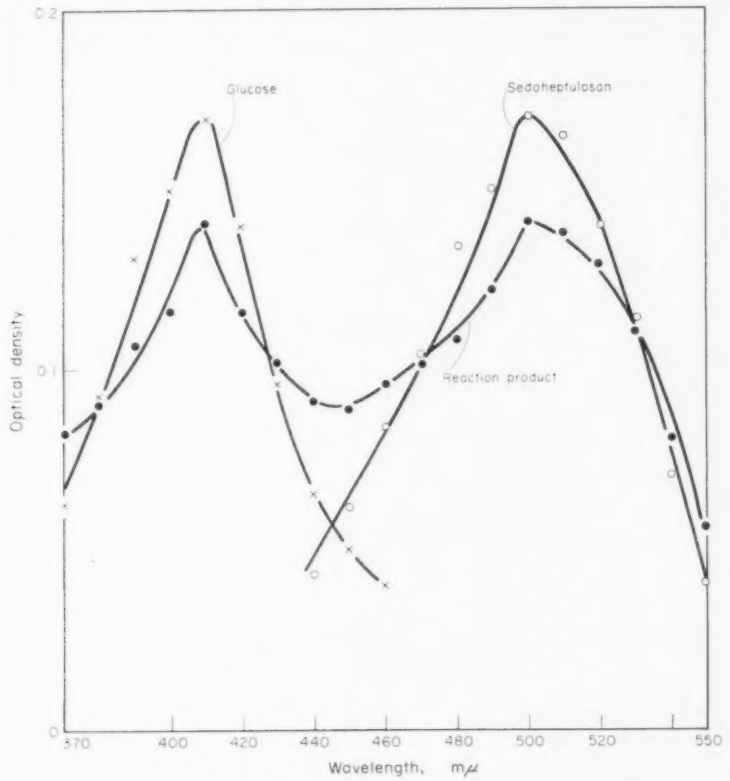

FIG. 6. The absorption spectra of the products of ribose-5-phosphate breakdown, sedoheptulose and glucose reacted with sulphuric acid–cysteine. For details see Table 3. The curve was corrected for endogenous absorption. Incubation time was 180 min.

hexose during the conversion of ribose-5-phosphate to hexose phosphate (Fig. 7) indicates the presence of transaldolase and that the sedoheptulose phosphate is most probably a substrate for transaldolase.

(2) *Stoichiometry*. The data in Table 3 indicate that heptulose and hexose (and triose, from the method of assay of transketolase) were the products of R-5-P breakdown. Furthermore, the amounts of intermediates seem to depend upon the length of incubation. The heptulose formed was identified as sedoheptulose after reaction with sulphuric acid–cysteine. The typical absorption

spectrum of the reaction products is seen in Fig. 6. The absorption spectrum of the reaction product exhibited two peaks at 415 mμ and 505 mμ corresponding to those of hexose and sedoheptulose.

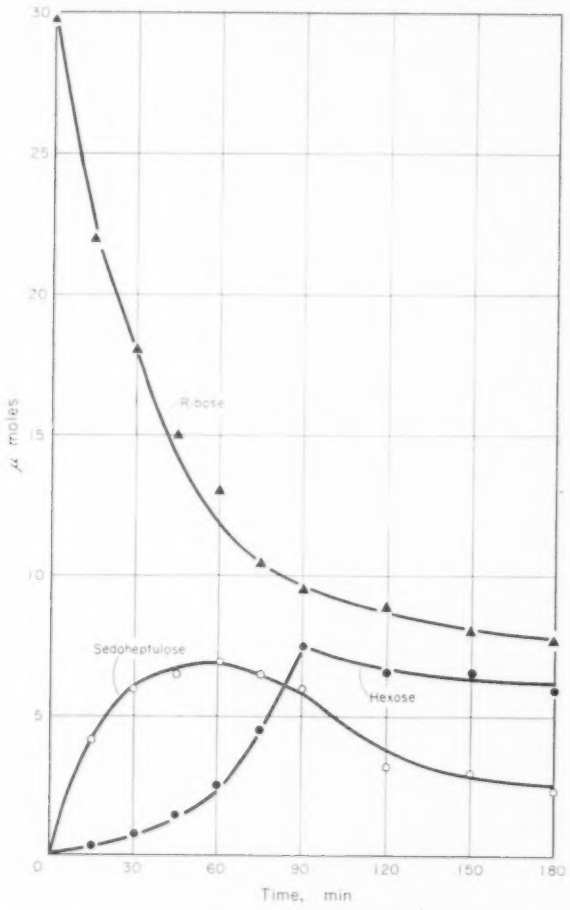

FIG. 7. Time course of ribose-5-phosphate utilization. For composition and treatment of the reaction mixture see Table 3.

The data in Table 3 suggest that during the first 30 min approximately 2 moles of pentose phosphate were utilized for each mole of heptulose formed, although only 80 per cent of the theoretical value of conversion was obtained in our experiments. After 30 min, however, if the value of hexose is also taken into consideration, the stoichiometry of 1 mole of heptulose and hexose phosphate arising from 2 moles of pentose phosphate was fully achieved.

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(3) *Time course of formation of products of ribose-5-phosphate breakdown.* The data in Table 3 indicate that hexose and sedoheptulose are the products of breakdown of ribose-5-phosphate. Moreover, the level of hexose phosphate and sedoheptulose phosphates formed appears to be a function of time. More information was therefore sought on the time interrelationship of the formation of these intermediates.

The data in Fig. 7 represent a typical sequence of events. Initially the utilization of ribose-5-phosphate is very rapid with approximately 40 per cent of the pentose disappearing in 30 min and about 70 per cent in 3 hr. At longer periods of incubation equilibrium is achieved. The participation of sedoheptulose as intermediate in the conversion of ribose-5-phosphate to hexose phosphate is indicated by the order in which the products appear. After 60 min, the level of sedoheptulose decreases, while the amount of hexose phosphate increases. Very little hexose monophosphate was formed during the early stages of incubation (less than 30 min). The properties of transaldolase, which are involved in the conversion of sedoheptulose phosphate to hexose phosphate, have been described by Horecker & Smyrniotis (1955). Furthermore, it has also been shown that an intermediate in this conversion is erythrose-phosphate (Horecker & Smyrniotis, 1955; Kornberg & Racker, 1955). No attempt was made to demonstrate the presence of erythrose phosphates in our preparation; however, it is likely that erythrose phosphate is formed in mites during the conversion of sedoheptulose to hexose.

DISCUSSION

In the present study the hexose monophosphate cycle, similar to that described for microorganisms, plants, insects and mammals, was found to occur in the two-spotted spider mite. The cyclic process consists of the following main steps: (a) two-step oxidation of glucose-6-phosphate to pentose phosphates and CO_2, (b) the conversion of pentose phosphates to sedoheptulose phosphates and triose phosphate, and (c) the conversion of sedoheptulose phosphate to hexose phosphate. These reactions in the mites were shown to be catalyzed by a number of enzymes, glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, phosphopentose isomerase, phosphoketopentose epimerase, transketolase and transaldolase.

Although ribulose-5-phosphate has been shown to be the product of 6-phosphogluconate oxidation in other organisms (Horecker *et al.*, 1951) it could not be shown in the present experiments. This was probably due to the presence of phosphopentose isomerase and phosphoketopentose epimerase in our preparation, which perhaps immediately converted the ribulose-5-phosphate formed to ribose-5-phosphate and xylulose-5-phosphate. And since the equilibrium of the reactions, as shown by Tabachnick *et al.* (1958), for the conversion of ribulose-5-phosphate to ribose-5-phosphate by phosphopentose isomerase and xylulose-5-phosphate by phosphoketopentose epimerase, is greatly towards the formation of ribose-5-phosphate and xylulose-5-phosphate, it reasonably explains the absence of ribulose and the presence of ribose and xylulose in the present experiments.

The demonstration of xylulose-5-phosphate in the present experiments is significant, because it has been shown to be an intermediate in the pentose metabolism in mammals, microorganisms, plants and insects (Ashwell & Hickman, 1955, 1957; Dickens & Williamson, 1956; Hochster, 1955; Stumpf & Horecker, 1956; Racker, 1956; Chefurka, 1958a) and has been shown to be a substrate for transketolase (Srere *et al.*, 1955).

Conversion of pentose to heptulose, in mite extracts, appeared to reach equilibrium only when 70 per cent of the pentose disappeared. Similar findings have been reported by Horecker & Smyrniotis (1955) and Glock (1952) for liver preparations and spinach transketolase, and by Chefurka (1958a) for the house fly enzyme. Also, there was a lag period, after which only the pentose conversion to sedoheptulose reached the theoretical value of 2 moles of pentose per mole of sedoheptulose. Essentially similar findings have been reported by Axelrod *et al.* (1953) for plants, Kinoshita & Masurat (1954) for corneal epithelium, and Chefurka (1958a) for the house fly.

The reversibility of the reactions of the hexose monophosphate cycle enzymes in the mite has not been shown, but it is most probable that these are reversible. Horecker & Smyrniotis (1952, 1953) have shown the reversibility of reactions catalyzed by glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase. Furthermore, reactions catalyzed by transketolase and transaldolase (Horecker & Smyrniotis, 1955; Horecker *et al.*, 1953) and phosphopentose isomerase and phosphoketopentose epimerase (Axelrod & Jang, 1954; Hurwitz & Horecker, 1956; Ashwell & Hickman, 1957; Tabachnick *et al.*, 1958) are also known to be reversible.

The main objective in this study has been to demonstrate the presence of the hexose monophosphate cycle in the two-spotted spider mite. In general, our results appear to indicate that all enzymes necessary for the operation of the cycle are present in these mites. Whether this cycle functions as a major energy source or serves to synthesize essential metabolites such as amino acids (Weissbach & Horecker, 1955) or keto acids (Tolbert & Cohan, 1953) has not been determined and must be investigated in the future.

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A COMPARATIVE STUDY ON VERTEBRATE BRAIN MITOCHONDRIA*†

VERA G. WAHBE, W. M. BALFOUR and F. E. SAMSON, Jr.

Department of Physiology, University of Kansas, Lawrence, Kansas, U.S.A.

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Abstract—Brain mitochondria from catfish, frog, turtle, chicken and rat were studied after separation by differential centrifugation. It was found that per gram of brain: (1) The wet weight of mitochondria ranges from 225 mg in the frog to 472 mg in the rat; (2) The dry weight of mitochondria ranges from 36 mg in the frog to 80 mg in the rat; (3) The mitochondrial protein ranges from 15 mg in the frog to 32 mg in the rat; (4) The oxidative capacity of rat brain is 10 times that of catfish brain; (5) The number of mitochondria ranges from $18{\cdot}8 \times 10^{10}$ in the frog to $33{\cdot}2 \times 10^{10}$ in The rat. The corresponding values for the turtle and the chicken were in the order of: frog, turtle, chicken and rat. With the exception of the catfish, the average mitochondrion for all the animals is strikingly similar with $1{\cdot}69 \times 10^{-12}$g wet weight, $0{\cdot}27 \times 10^{-12}$g dry weight and $0{\cdot}11 \times 10^{-12}$g protein.

INTRODUCTION

IN BRAIN tissue, the mitochondria may be considered the "power-houses" of the cells as they are in the other organs. That is, they contain the complete mechanisms for the conversion of the energy which reaches the brain in the form of food into forms that can be used by the cellular machinery. It might be expected that differences in the metabolic activity of various tissues would be reflected in a number of characteristics of the mitochondria, as seen, for example, in the great increase in the number of mitochondria during the development of the neonatal rat brain (Samson *et al.*, 1960). The difference in the energy metabolism of the brain in various vertebrate animals suggested this comparative study of vertebrate brain mitochondria. The question asked was: Does the number, size or oxidative activity of the brain mitochondria differ in animals from various classes of vertebrates ?

METHOD

Whole brains of adult animals were used for all experiments. The brain had to be large enough to yield sufficient quantities of mitochondria, yet not so large as to present problems in decapitation and quick extraction. *Ictalurus melas* (black bullhead catfish), *Rana pipiens* (grass frog), *Pseudemys elegans* (Cumberland terrapin), *Gallus gallus* (domestic fowl), and *Mus norwegicus albinus* (albino rat) were used in the experiments.

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† Abridgement of a thesis submitted by Miss V. Wahbe to the Graduate School of the University of Kansas in partial fulfilment of the requirements for the degree of Master of Arts in Physiology.

The animals were decapitated, the cranium opened, and the brain removed as quickly as possible. The brain was placed in about 30 ml of 0·25 M sucrose and 0·01 M EDTA (Ethylenedinitrilo Tetraacetic Acid Disodium Salt) solution at a pH of 7·4 and cooled to a temperature between 0° and 5°C. The excess liquid was removed from the brains by placing them on filter paper and blotting them gently. The brain tissue was weighed on a Roller-Smith balance and then homogenized in 40 ml of the sucrose-EDTA solution with a Teflon pestle in a glass homogenizer

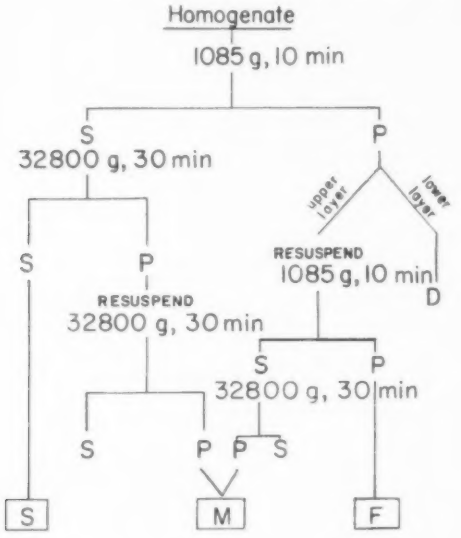

FIG. 1. Centrifugation scheme. P = pellet; S = supernatant.

(clearance 0·006–0·009 in.), with 15 passes of the pestle. The homogenate was centrifuged at a sedimentation constant $(S) = 9{\cdot}7 \times 10^4$ (1085 g for 10 min) in a Servall model SS-I angle head refrigerated centrifuge. (S) describes the force on the average particle and is calculated from the formula:

$$(S) = 3{\cdot}5 \frac{\left[\log\left(\frac{R_{max}}{R_{min}}\right)\right](10^{13})}{\int_0^t (\text{r.p.m.})^2\, dt},$$

where R_{max} and R_{min} are radii of circles described by the particles which are at a maximum and minimum distance from the axis of the centrifuge head, t is time in minutes, and r.p.m. is revolutions of the head per minute (de Duve & Berthet, 1953). Fig. 1 shows the centrifugation scheme followed. The initial pellet was made up of 2 parts: a top layer, which was cream colored and lightly packed, and a bottom layer which had a red tinge and was firmly packed. The top layer was very gently scraped off with a rubber policeman. This layer contains the first mitochondria to come

down. The firm lower layer, *D*, made up of nuclei, cell walls and debris, was discarded. The upper layer of the pellet was resuspended in 40 ml of the sucrose-EDTA solution by 8 passes of the pestle. The suspension was centrifuged at $(S) = 9{\cdot}7 \times 10^4$ (1085 g for 10 min) and the pellet obtained was resuspended by 8 passes of the pestle in several ml of the cold sucrose-EDTA solution to make the *F*-fraction. The supernatant was centrifuged at $(S) = 1{\cdot}2 \times 10^3$ (32,800 g for 30 min) and a mitochondrial pellet was obtained. The supernatant of the first slow spin was also centrifuged at $(S) = 1{\cdot}2 \times 10^3$ (32,800 g for 30 min) and a cream colored mitochondrial pellet appeared. The supernatant of this pellet made up the *S*-fraction. The pellet was washed by resuspension in 40 ml of the sucrose-EDTA solution and then recentrifuged at $(S) = 1{\cdot}2 \times 10^3$ (32,800 g for 30 min). The 2 mitochondrial pellets were resuspended together in several ml of the sucrose-EDTA solution to make the *M*-fraction. From this centrifugation procedure 3 fractions (*M*, *F* and *S*) were obtained upon which the studies were made.

For the *M* and *F*-fractions wet weight, dry weight, protein, oxidative capacity and mitochondrial counts were determined. Only oxidative capacity and counts were determined for the *S*-fraction. For the wet and dry weight duplicate aliquots of 1·0 ml each were transferred to tared centrifuge tubes, and centrifuged at $(S) = 1{\cdot}96 \times 10^3$ (32,800 g for 20 min) in 0·9% KCl. The supernatants were poured off and the tubes plus the pellets were weighed. The pellets were then transferred to tared flasks and dried overnight at 110°C. The flasks were again weighed and the dry weight calculated.

The mitochondrial protein was precipitated with 5% perchloric acid and the lipids removed by successive extraction with 0·1 N potassium acetate in ethanol and iso-propyl ether (Lowry *et al.*, 1951). The protein was determined by the biuret reaction as modified by Aldridge (1957) with bovine serum albumin as reference standard.

To determine the oxidative capacity, the reduction of 2,3,5-Triphenyl Tetrazolium Chloride (TTC) was used (Kun & Abood, 1949). The reaction mixture contained 1 ml 0·1 M phosphate buffer pH 7·2, 0·5 ml of 0·5% TTC, 2·5 ml 0·02 M succinate and 1 ml of sample. The control tubes contained the same reagents plus 1 ml of glacial acetic acid to stop the activity of the mitochondria. All the tubes were incubated at 37°C for 1 hour and 1 ml of glacial acetic acid was then added to each sample tube. The red color of the reduced TTC was extracted in 0·5 ml acetone and 5 ml toluene with vigorous shaking. The top layer was centrifuged for several minutes and its optical density was read at 460 mμ on a Beckman spectrophotometer. Values for reduced TTC are in reference to a standard curve made by reducing known concentrations of TTC solutions with $Na_2S_2O_4$.

To determine the number of mitochondria a volume of mitochondrial suspension from a known amount of brain was diluted to a known volume in 1·8 M sucrose and counts were made in a Petroff-Hauser bacteriological counting chamber with a phase microscope. The high concentration of sucrose was used as suggested by Allard *et al.* (1952).

RESULTS

The values for wet and dry weight and protein content of mitochondria in a gram of brain from various animals are presented in Table 1. The weight of mitochondrial material differs significantly among the animals compared. The catfish has high values which are close to rat values. The per cent water in the

TABLE 1—COMPOSITION OF THE *M*-FRACTION MITOCHONDRIA

Animal	No. animals/ No. experiments	Wet wt. mg/g brain Mean ± S.E.	Dry wt. mg/g brain Mean ± S.E.	Protein mg/g brain Mean ± S.E.	Per cent H_2O	Per cent protein of dry wt.
Catfish	8/2	456 ± 45·1	77 ± 11·2	24.5 ± 0·5	83	32·4
Frog	20/2	225 ± 21·0	36 ± 5·2	15·3 ± 2·2	84	44·3
Turtle	6/3	308 ± 21·9	46 ± 2·8	19·7 ± 0·5	85	43·1
Chicken	3/3	348 ± 5·2	54 ± 2·3	22·0 ± 1·2	84	40·6
Rat	3/3	472 ± 7·7	80 ± 2·9	32·3 ± 5·7	83	40·4

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mitochondria (Table 1) is similar in the five animals, varying only from 83 per cent in the catfish and rat to 85 per cent in the turtle. The per cent of the dry weight which is protein is also similar with the exception of the catfish value, which is about 10 per cent less than for the other animals.

TABLE 2—OXIDATIVE CAPACITY: MICROMOLES OF TTC REDUCED PER HOUR PER GRAM BRAIN

Animal	No. animals/ No. experiments	*M*-fraction	*M*-per cent of total	*F*-fraction	*S*-fraction	*F*+*M*+*S* fractions Mean ± S.E.
Catfish	8/2	0·34	87	0·028	0·019	0·39 ± ·18
Frog	20/2	0·95	90	0·106	0·004	1·06 ± ·31
Turtle	6/3	1·97	99	0·009	0·007	1·99 + ·21
Chicken	3/3	2·27	77	0·663	0·004	2·94 ± ·37
Rat	3/3	3·55	95	0·192	0·007	3·75 ± ·18

The oxidative capacity which is indicative of the energy production of the mitochondria increases tenfold from catfish to rat per gram of brain (Table 2). The number of mitochondria increases from frog to rat (Table 3).

DISCUSSION

It is clear from these observations that the procedures used to prepare mitochondria from mammalian liver applied to brain tissue of animals representing the major classes of vertebrates will yield material which has properties attributed to typical mammalian mitochondria. That is: (a) The general appearance of the

sedimented material is the same in all animals studied; (b) particles from the different brains possess striking resemblance under phase-microscopy; (c) the mitochondrial fraction rapidly metabolizes sodium succinate. If succinic dehydrogenase is found only in mitochondria as shown for liver mitochondria by Hogeboom *et al.* (1948), then the major part of the cerebral mitochondria in vertebrates

TABLE 3—NUMBER OF MITOCHONDRIA PER GRAM BRAIN

Animal	No. animals/ No. experiments	Supernatant	*F*-fraction	*M*-fraction	*M*-per cent of total	Total Mean ± S.E.
		$\times 10^{10}$	$\times 10^{10}$	$\times 10^{10}$		$\times 10^{10}$
Catfish	8/2	2·1	2·7	17·2	78	22·0 ± 2·2
Frog	20/2	0·2	5·3	13·2	71	18·8 ± 2·6
Turtle	6/3	1·3	4·6	16·8	74	22·7 ± 2·8
Chicken	3/3	0·2	6·3	22·5	78	28·9 ± 0·4
Rat	3/3	0·3	5·1	27·9	84	33·2 ± 0·8

appears in the *M*-fraction of the differential centrifugation procedure used here. Indeed, essentially all of the succinic dehydrogenase activity was in the *M*-fraction (see Table 2) in the turtle and in the rat. On the basis of phase-microscopy identification, 70–80 per cent of the mitochondria were in the *M*-fraction (Table 3).

TABLE 4—CHARACTERISTICS OF AN AVERAGE MITOCHONDRION FROM THE *M*-FRACTION

Animal	No. animals/ No. experiments	Wet weight (pg) Mean ± S.E.	Dry weight (pg) Mean ± S.E.	Protein (pg) Mean ± S.E.	(10^{-18} moles) TTC reduced per hour Mean ± S.E.
Catfish	8/2	2·6 ± ·02	0·445 ± 0·017	0·144 ± 0·014	2·1 ± 1·2
Frog	20/2	1·7 ± ·40	0·284 ± 0·08	0·115 ± 0·001	7·5 ± 2·1
Turtle	6/3	1·9 ± ·19	0·279 ± 0·027	0·121 ± 0·014	11·7 ± 0·3
Chicken	3/3	1·6 ± ·26	0·257 ± 0·016	0·104 ± 0·023	10·9 ± 2·7
Rat	3/3	1·7 ± ·06	0·287 ± 0·015	0·117 ± 0·023	10·4 ± 1·5

pg = 10^{-12} g

The higher per cent of succinic dehydrogenase in *M* over the per cent mitochondria seen under phase-microscopy may be due to greater dehydrogenase activity per particle in *M* than in the *F*-fraction or to particles being counted as mitochondria in *F* which did not have succinic dehydrogenase activity. In any event, it seems clear that most of the mitochondria come down in the *M*-fraction.

If we can assume that the only particles in *M* are those identified as mitochondria by phase-contrast microscopy, then it is possible to calculate an average dry weight, protein and water content of an individual mitochondrion from the data in Tables

1 and 3. The results of such a calculation are given in Table 4 as the characteristics of an average mitochondrion. With the exception of the catfish, the generalization emerging from these characteristics is the striking similarity in the mean wet weight, dry weight and protein content of a cerebral mitochondrion from all the animals.

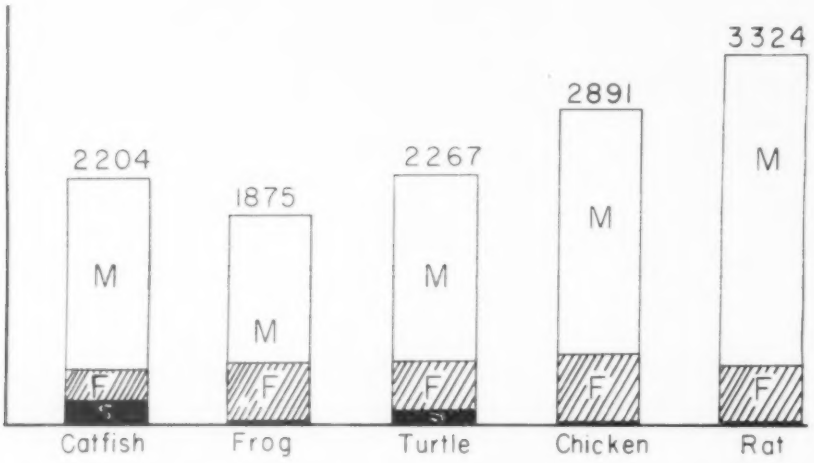

FIG. 2. Mitochondrial census ($\times 10^8$) per gram brain. *S* represents the number of mitochondria in the supernatant (black area). *F* represents the number of mitochondria in the *F*-fraction, mitochondria that sedimented at slow centrifugation. *M* represents the number of mitochondria in the *M*-fraction, mitochondria sedimented at fast centrifugation. The number at the top of each bar graph represents the total of *F*, *M* and *S* fraction counts.

Although the above characteristics are similar, the capacity to reduce TTC with succinate as a substrate is not, the capacity per mitochondrion in the rat being five times that in the catfish. It is evident that the increased capacity per mitochondrion is not accounted for by any great change in protein content.

Fig. 2 shows the main increase in mitochondrial number to be in the *M*-fractions. The number of mitochondria in the *F*-fraction, on the other hand, does not seem to vary widely in the different animals. The catfish has more mitochondria than does the frog, but, as mentioned earlier, the oxidative capacity is lower than that of the frog.

The number of cells per gram of brain was calculated for the rat and the chicken based on the amount of deoxyribose nucleic acid (Vendrely, 1955). The average number of mitochondria per cell in the rat is 1440, and in the chicken is about 800. If some measure of the average dendritic complexity could be made, it is possible that the number of mitochondria would be correlated with the area of active surface since the energy for maintaining chemical gradients might be expected to be directly related to the size of the active surfaces.

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AQUATIC RESPIRATION IN THE COMMON NILE TURTLE, *TRIONYX TRIUNGUIS* (FORSKÅL)

SABET GIRGIS

Zoology Department, University of Khartoum, Sudan

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Abstract—(1) The anatomy of the oral respiratory system, i.e. villiform processes, pharyngeal blood supply, of the turtle *Trionyx* is described.

(2) The oxygen consumption as determined by the Winkler method showed that the quiet submerged animal used up 6·5 ml oxygen/kg/hr. Pharyngeal respiration used up 2·04 ml oxygen/kg/hr.

(3) Pharyngeal respiration thus accounted for approximately 30 per cent of the respiration of the quiet submerged animal; the other 70 per cent being due to cutaneous respiration.

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A. INTRODUCTION

AGASSIZ (1857) measured the lung capacity of turtles in relation to their weight and in association with their habits. He found that in *Amyda ferox* lung capacity to body weight was in the ratio 1 : 17; in *Cinosternon pennsylvanicum*, which is also mainly aquatic, it was 1 : 16; in *Cistudo* (*Terrapene*) *triunguis*, a mainly terrestrial species, the ratio was almost 1 : 1. Hence aquatic turtles have proportionately much smaller lung capacity than have terrestrial species.

Gage and Gage (1886) described filamentous villiform processes in *Amyda spinifera* and *A. mutica* which apparently function as gills. They found that the mucous membrane of the pharynx is highly vascular, the capillaries lying in the processes. They also showed that there is a rhythmic movement of the hyoid apparatus at the rate of 16 pulses/min. By raising and lowering this structure, the mouth and pharynx are filled with water and then emptied.

Gadow (1887) described "paired anal pouches" which "are present in the amphibiotic Emydes and in the more aquatic Chelydae but absent in the terrestrial Chersidae and in the marine Cheloniidae. In most mud tortoises (Trionychidae) they seem likewise to be absent." Water is passed in and out of the cloaca to the anal pouches that act as accessory respiratory organs.

Cahn (1937) demonstrated pharyngeal respiration in the soft-shelled turtle *Amyda mutica* and the hard-shelled *Chrysemys* and *Pseudemys* (species not given) by inserting grains of carmine in the water and observing their sudden movement into the nasal passage as the hyoid apparatus drops. He pointed out that a similar phenomenon can also be demonstrated at the anus.

Krogh (1941), however, rules out throat movements as having a respiratory function and considers them to be olfactory. McCutcheon (1942) also considers these movements to be associated primarily with olfaction. Root (1949) compares

oxygen consumption of the musk turtle *Sternotherus odoratus* when the animal was completely submerged with normal oxygen consumption in the air.

Specimens required for this work were caught near Khartoum from both the Blue Nile and the White Nile. A special turtle-pond, to keep the animals alive,

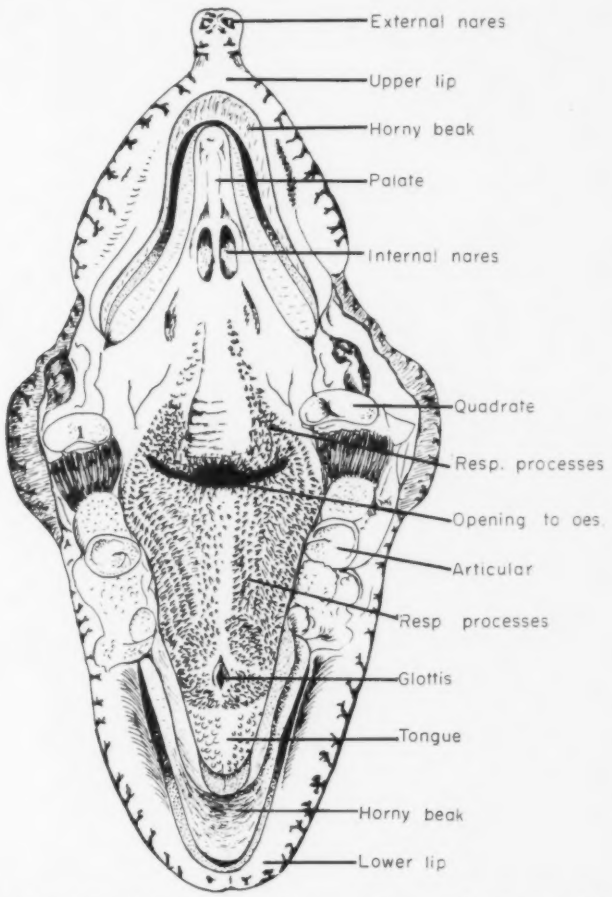

FIG. 1. The upper and lower jaws have been forced open to show the distribution of the villiform processes in both the roof and the floor of the mouth and pharynx. The specimen is placed under water.

was constructed in the grounds of the University of Khartoum. The animals were fed once a day on fish, dead and alive, and on the flesh of animals dissected for class work. The turtles could be watched while swimming, mating or lying quietly under water, sometimes for hours. Occasionally some buried themselves in the sand and remained fasting for several days.

B. ANATOMY

A very great number of minute hair-like processes cover practically the entire mucous membrane of the floor of the pharynx. They are easily observed when the lower jaw of a freshly killed turtle is severed from the animal and placed under water. The villiform processes float and can then be seen as short unbranched cylinders or cones which lie very close together but separate from one another.

When the upper and lower jaws are forced open and placed under water (Fig. 1) the processes are seen to lie not only in the mucous membrane of the pharyngeal floor, but also in that of the roof where they are confined to a small area near the opening of the oesophagus.

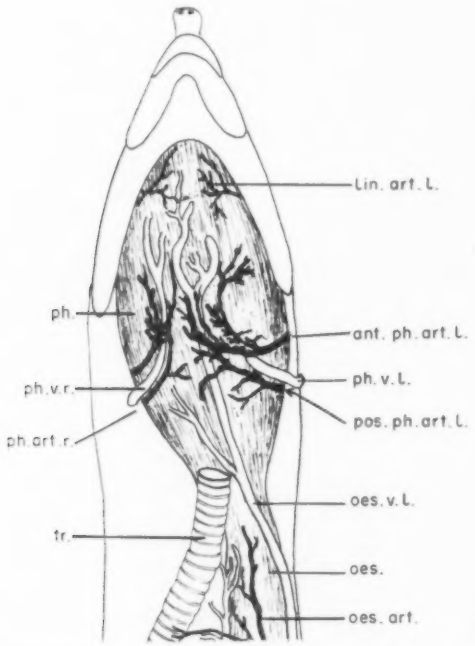

FIG. 2. Ventral dissection to show the blood vessels in the floor of the mouth and pharynx. ant. ph. art. l., left anterior pharyngeal artery; lin. art. l., left lingual artery; oes., oesophagus; oes. art., oesophageal artery; oes. v.l., left oesophageal vein; ph., pharynx; ph. v.l., left pharyngeal vein; ph. v.r., right pharyngeal vein; pos. ph. art. l., left posterior pharyngeal artery.

The respiratory processes can be distinguished from the taste papillae found on the tongue and other parts of the mucosa of the mouth and pharynx by the obvious difference in shape, and also because the former are swayed easily by any movement of the water covering them. They are densely crowded around the glottis but practically none are found in a narrow ribbon in the mid-longitudinal axis of the

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pharyngeal floor. Again, they are particularly numerous in the area on either side of the ribbon where they appear to be arranged in close parallel rows.

The pharyngeal wall which supports the respiratory processes is very highly vascular. The blood vessels supplying the area break into a dense network, a rete mirabile. The wall receives on either side one and sometimes two pharnygeal arteries (Fig. 2) from the corresponding common carotid arteries (not shown in the figure, as they are somewhat dorsal). It is drained mainly by the right and left pharyngeal veins (Fig. 2), opening into the right jugular vein which extends along the mid-dorsal aspect of the anterior part of the neck. To some extent the pharyngeal wall is also drained by tributaries of the left oesophageal vein (Fig. 2) which runs along the ventral wall of the mouth and pharynx to the left side of the oesophagus and opens into the left jugular vein.

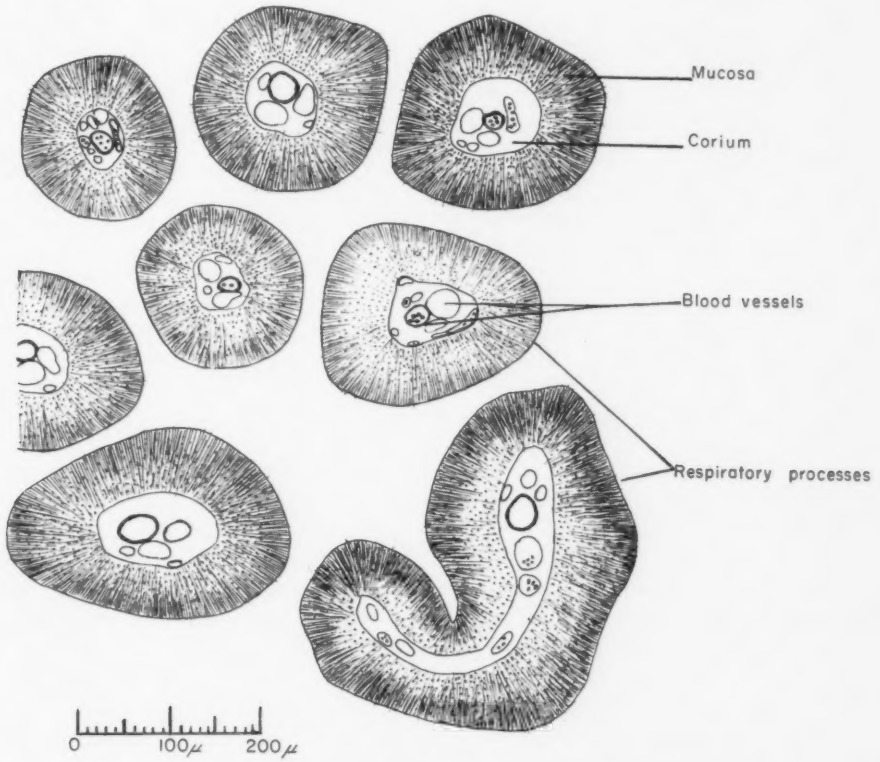

FIG. 3. Diagram of a section through a number of respiratory processes.

C. HISTOLOGY

Sections of the pharyngeal mucosa were cut and stained with haematoxylin-eosin, and with Mallory's triple stain. The low power drawing (Fig. 3) shows some processes cut more or less transversely.

Fig. 4 is a camera-lucida drawing of a T.S. in a villiform process made under high power. The stratified epithelium of the mucosa contains two or three layers of elongated cells. Typical mucous cells are seen scattered in the outer layer. Intercellular spaces exist between some of the cells which are not densely crowded except in the outer layer. A large number of small lymphocytes are found all over the mucous coat and a few are also found in the corium. The inner layer of cells rests on a thin but clear basement membrane.

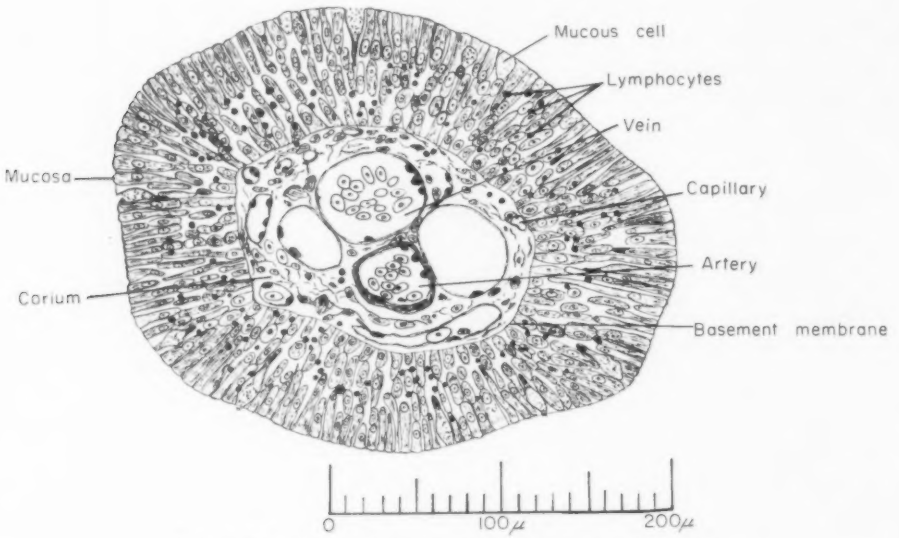

FIG. 4. T.S. of a respiratory process. Camera-lucida drawing.

The loose connective tissue of the corium shows a few fibres and some scattered connective tissue cells. It contains a large number of vessels of various diameters.

D. EXPERIMENTAL

Experiments were performed to demonstrate the existence of aquatic respiration and to determine the part played by the pharyngeal processes, since their anatomy and histology suggest a respiratory function.

Methods

Oxygen consumption through various regions of the body was estimated. Experiments were carried out in two all-glass tanks of 27 and 23·8 l. capacity. Tap water and Nile water were both employed in the preliminary experiments, but as no difference was observed in the results obtained tap water only was used in later experiments.

Preliminary experiments were carried out for periods of 2, 3, 4, 5 and 6 hr to ascertain the most suitable period. Some animals died and many were restless or

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stayed motionless in the water when the experiments lasted for over three hours. The turtle sometimes lost its balance, and extended its neck and limbs. Some individuals were drowned because of the entrance of water into their lungs. The experiments described below were therefore carried out for 3 hr. The behaviour of the animal was observed during each experiment.

Four specimens were used, their weights being 605 g, 1110 g, 1270 g and 1520 g. Larger sizes could not be used because the experimental tanks were not big enough. All four turtles were kept in the laboratory and fed once daily. Minced fish or meat was placed in the tank at 9.00 a.m. The hungry animals would dash at it.

TABLE 1—AQUATIC RESPIRATION OF *Trionyx triunguis*; ENTIRE ANIMAL. TEMPERATURE: 23–24°C

Expt. No.	Oxygen consumption (ml/kg/hr)
1	7·2
2	7·4
3	6·9
4	7·3
5	6·5
6	6·3
7	6·1
8	7·1
9	6·7
10	6·9

Average oxygen consumption = 6·8 ± 0·6 ml/kg/hr

Remains of food were removed and the water was changed after 20 min. Experimental animals were given a shower, then immersed in a bath of potassium permanganate and washed in running tap water before being introduced into the experimental tank. This was then completely filled with water and covered with a glass sheet, special care being taken to exclude air bubbles. Oxygen content was estimated by Rideal and Stewart's modification of Winkler's method (1901). Two water samples were taken for analysis both before and after termination of each experiment and the mean result recorded. Differences, if any, between samples were slight, as the water was continuously stirred by the movement of the turtle. The animal was weighed, and mean temperature of the water recorded.

Ten measurements were made in each of the following experiments:

1. Determination of aquatic oxygen uptake (Table 1).

2. Determination of aquatic oxygen uptake from animals with the cloacal region covered (Table 2). The tail region was covered with a rubber sheath to determine the effect, if any, of anal respiration.

3. Determination of aquatic oxygen uptake by means of buccopharyngeal respiration (Table 3). The body of the turtle was covered with a tight cloth covered with a nylon sac. The cloth protected the nylon sac from the sharp

claws of the turtle. The head and neck were kept free by means of a rubber-metal ring which prevented the withdrawal of the neck into the sac.

TABLE 2—AQUATIC RESPIRATION OF *Trionyx triunguis*; CLOACAL OPENING COVERED. TEMPERATURE: 23–24°C

Expt. No.	Oxygen consumption (ml/kg/hr)
1	6·13
2	7·16
3	5·58
4	5·69
5	6·83
6	6·39
7	6·56
8	6·29
9	6·72
10	5·96

Average oxygen consumption = 6·3 ± 0·86 ml/kg/hr

TABLE 3—AQUATIC RESPIRATION OF *Trionyx triunguis*; BUCCOPHARYNGEAL REGION ALONE (BODY COVERED WITH A NYLON SAC). TEMPERATURE: 23–24°C

Expt. No.	Oxygen consumption (ml/kg/hr)
1	1·63
2	2·38
3	1·73
4	2·38
5	1·95
6	1·79
7	1·86
8	2·00
9	2·49
10	2·17

Average oxygen consumption = 2·04 ± 0·45 ml/kg/hr

4. Determination of the aquatic oxygen uptake through the buccopharyngeal region and body separately (Table 4). The experimental tank was divided into two unequal compartments one for the head and neck and the other for the body of the turtle. The two compartments were separated by a metal sheet in the middle of which was a hole for the neck. The neck was kept in position and prevented from being withdrawn by means of a metal-rubber ring which also prevented mixing of the water in the two compartments, The experimental tank of 23·8 l. capacity was used and the two compartments contained 5·5 and 18·3 l. respectively.

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Calculation

The initial and final oxygen concentrations were calculated. The difference gives the change in oxygen concentration during the period of the experiment in $g/10^5$ and ml/l. The weight of the animal used was recorded and the oxygen consumption in ml/kg/hr was calculated according to the equation given by Root (1949), i.e.

$$\frac{1000 \times RO}{GT}$$

where R = respirable volume in litres
O = difference between initial and final oxygen concentrations of water
G = weight of animal in grammes
T = experimental time in hours.

The respirable volume of water was determined after deducting the volume of the turtle from the volume of the water (or part of turtle from volume of compartment in experiment 4). The experimental results are given in tables showing the oxygen consumption per kg/hr.

TABLE 4—AQUATIC RESPIRATION OF *Trionyx triunguis*; BUCCOPHARYNX AND CUTANEOUS SEPARATELY. TEMPERATURE 28°C

Expt. No.	Buccopharyngeal Oxygen consumption (ml/kg/kr)	Body Oxygen consumption (ml/kg/hr)
1	1·32	4·39
2	1·25	4·34
3	0·98	4·46
4	0·98	4·51
5	0·88	3·67
6	1·22	4·19
7	0·92	3·31
8	1·29	4·62
9	0·96	3·52
10	1·09	4·08
	Average 1·09 ± 0·23 ml/kg/hr	Average 4·11 ± 0·70 ml/kg/hr

Behaviour of an animal during a typical experiment

The behaviour of the turtle during each experiment was recorded, and in a typical experiment it was as follows:

As soon as the tank was closed the animal started to swim vigorously, tilting its body in all directions. After 20 min movement stopped except for rhythmic pulsations of the hyoid apparatus at a rate of 6/min. After 5 min vigorous movements began again and persisted for another 5 min. All general movement then stopped and pulsation of the hyoid apparatus took place at the rate of 6–10/min. Periods of activity alternated with periods of rest until the end of the experiment, but general

movement became less and occurred after longer intervals. Movements of the hyoid varied from 0–6/min for intervals of up to 12 min. During these intervals, the animal stretched its neck and limbs and was entirely motionless. Sometimes it lost its balance and lay upside down or on its side.

Clearly the animal behaved in a peculiar and unnatural way during the experiment, being disturbed at the outset and exhausted towards the end.

RESULTS

(1) Table 1 shows that aquatic respiration does, in fact, take place.

Oxygen consumption was $6{\cdot}8 \pm 0{\cdot}6$ ml/kg/hr, at a temperature of 23°C–24°C.

(2) Table 2 shows that aquatic respiration with the cloaca covered is practically the same as in the first set of experiments. Oxygen consumption was $6{\cdot}3 \pm 0{\cdot}86$ ml/kg/hr, at a temperature of 23°C–24°C. The difference is too slight to require comment. It may therefore be concluded that in *Trionyx* there is very little or no cloacal respiration.

(3) This set of experiments is of special importance because any aquatic respiration here will be due to pharyngeal respiration alone. Oxygen consumption was $2{\cdot}04 \pm 0{\cdot}45$ ml/kg/hr, at a temperature of 23°C–24°C.

This is about 30 per cent of the total aquatic respiration.

This series of experiments shows that the turtle respires through its pharynx as well as through its skin, and that pharyngeal respiration is less than cutaneous respiration.

DISCUSSION

There seems to be little doubt that aquatic turtles satisfy part of their oxygen requirements from the water they live in. This accessory respiration would, according to Agassiz (1857), compensate for the smaller lung capacity of aquatic species as compared to terrestrial ones.

Root (1949) found that, in *Sternotherus odoratus*, total aquatic oxygen consumption was equivalent to about one-eighth of that in air. The buccopharyngeal fraction was 32 per cent of the total aquatic oxygen consumption while the remainder was by absorption through the skin. Root concluded that aquatic respiration, particularly the buccopharyngeal fraction, contributes insignificantly to the ability of these animals to remain submerged.

It is clear that in their normal life soft-shelled turtles show two extremes:

(a) excessive activity as noticed and commented on by Agassiz (1857) who described their swimming movements, and Garman (1890) who reported that a turtle can pursue and catch the quickest of fish and can swim against swift currents and dash away in danger;

(b) a state of inactivity in which they lie quietly under water, for hours on end, completely motionless except for the regular up and down movements of the hyoid and the occasional closing of the eyelids. A close watch was kept on the turtles in the University pond and it was found that they may remain submerged for at least 6 hr.

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It seems therefore that Root's (1949) conclusion that "aquatic respiration contributes insignificantly to the ability of turtles to remain submerged" cannot be justified when the animals are in a state of inactivity and their rate of metabolism has dropped to a minimum. The little oxygen they get from the water is adequate for their modest demand. As for active animals, they cannot possibly survive on the limited supply of oxygen which they can get through aquatic respiration. This view is also confirmed by Cahn (1937) who states that "aquatic respiration is not sufficient for the turtles if they are active which explains why softshelled turtles die so readily when caught in net". Preliminary experiments in the present work likewise showed that few specimens could survive for more than 6 hr in experimental tanks when access to air was denied.

Gage and Gage (1886) showed that the rhythmic movements of the hyoid apparatus of *Amyda spinifera* and *A. mutica* occurred at an average rate of 16 movements/min. In *Trionyx* living at ease in the University pond the rate was 14–16/min. Yet under the unnatural conditions of the experimental tank, the number decreased gradually to 6 movements/min and sometimes stopped altogether for intervals which varied in length. Thus hyoid movements appear to slow down at moments of stress when the animal is much in need of oxygen, although this is the sort of situation in which one would expect emergency mechanisms to operate.

Krogh (1941) considered hyoid movements to be entirely olfactory. Evidence from the present experimental work, as well as from a study of the anatomy and histology of the villiform processes of the pharynx, shows that some aquatic pharyngeal respiration takes place. The movements which empty and fill the mouth and pharynx, therefore, serve a respiratory function. Nevertheless, there is good reason to believe that they are at the same time also olfactory. As shown by McCutcheon (1942) hyoid movements speed up when food is brought near the nose of an animal. Root (1949) showed that throat movements are irregular and do not increase at the early stages of respiratory stress. He concluded that these throat movements subserve primarily a chemical or olfactory function and their respiratory function is a secondary one.

Finally, it is important to realize that the region of the pharynx which contains the respiratory processes gets its blood supply from the common carotid arteries. Thus, capillaries in the processes contain oxygenated blood which does not require to be further oxygenated unless its oxygen saturation has dropped through the absence of air breathing, as happens when the turtle remains submerged for a long period. Therefore it seems that both the elaborate branching of the carotids into a rete mirabile in the pharyngeal wall, and the pharyngeal respiratory processes are functional adaptations which enable turtles to lie *quietly* under the water for long, but not indefinite periods.

SUMMARY

(1) In the turtle *Trionyx triunguis* dense villiform processes cover the wall of the pharynx.

(2) This wall receives pharyngeal arteries from the *common carotid* arteries, the former breaking into a rete mirabile.

(3) Sections in the processes show a mucous coat, surrounding a corium of loose connective tissue.

(4) The mucous coat consists of two to three layers of epithelial cells, not densely crowded, and containing mucous-secreting cells and lymphocytes.

(5) The loose connective tissue of the corium supports a large number of blood vessels of various sizes.

(6) Turtles were confined for 3 hr in experimental tanks completely filled with water. Initial and final oxygen contents were determined by Rideal and Stewart's modification of Winkler's method (1901).

(7) Oxygen consumption for the duration of the experiment was calculated in $g/10^5$ and ml/l.

(8) Oxygen consumption per kilogram of body weight per hour was calculated according to Root's (1949) equation.

(9) Ten experiments were performed for each of the following types, and the results tabulated:

1.—Aquatic respiration for the whole animal.

2.—Aquatic respiration with the cloacal opening covered.

3.—Aquatic respiration with the body covered (only the head and part of neck exposed).

4.—Aquatic respiration with head and body in separate chambers.

(10) Results of experimental work show that some aquatic respiration occurs, the buccopharyngeal fraction being about 30 per cent and the remainder through the skin. Cloacal respiration is negligible.

(11) A general discussion follows. Aquatic respiration does not meet the needs of active turtles, but seems to be adequate for inactive ones. Pharyngeal respiratory processes receive oxygenated blood from the *carotid* arteries. The blood is unlikely to be oxygenated from the surrounding water unless aerial respiration stops for some time. This takes place when turtles remain submerged for long periods.

Acknowledgements—The writer is very deeply indebted to his colleague, Dr. H. M. Bishay, for preparing the various solutions used throughout the investigation and for making the calculations. Thanks are also due to Prof. H. Sandon for encouragement, to Prof. J. E. Smith for his advice and to Prof. J. L. Cloudsley-Tompson for reading the manuscript.

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SHORT COMMUNICATION

EURYHALINITÉ ET FRÉQUENCE CARDIAQUE CHEZ LES TÉLÉOSTÉENS

A. SERFATY et R. LABAT

Laboratoire de Biologie Animale, Faculté des Sciences,
38 Rue des Trente-six Ponts, Toulouse (Haute-Garonne), France

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Résumé—Les auteurs ont opéré sur des espèces présentant des modes de vie très différents, à savoir *Anguilla anguilla* L., *Mugil capito* C. et V., *Mugil auratus* Risso, *Cyprinus carpio* L. et *Cantharus cantharus* C. et V.

Les passages eau de mer–eau douce, ou inversement eau douce–eau salée, ont été réalisés dans l'aquarium même où se trouvaient les individus afin d'éviter tout réflexe aéro-cardiaque dû au contact de l'air. Lors des variations de salinité il semble que l'effet chronotrope soit d'autant plus important que l'individu est plus sténohalin. Chez ces derniers les modifications rapides et brutales de la fréquence cardiaque, tachycardie suivie d'une bradycardie, entraînent la mort des sujets.

Abstract—(1) The authors have studied species possessing very different types of life: *Anguilla anguilla* L., *Mugil capito* C. et V., *Mugil auratus* Risso, *Cyprinus carpio* L. and *Cantharus cantharus* C. et V.

(2) The transfers from sea water to fresh water, and inversely from fresh water to sea water, have been carried out in the aquarium itself which contained these species, in order to avoid any airo-cardiac reflex, due to contact with the air.

(3) With these variations in the quantity of salt in the water, it would seem that the more the species is stenohaline, the more important is the corresponding chronotropic effect. With the stenohaline species the rapid and extreme modifications of cardiac frequency, tachycardia followed by a bradycardia, bring about the death of the subject.

Un poisson peut être comparé dans une certaine mesure à un osmomètre dont la membrane est représentée par les branchies et la peau (recouverte ou non d'écailles, avec ou sans mucus).

Le poisson se trouve dans le milieu extérieur qui est soit de l'eau de mer, soit de l'eau saumâtre, soit de l'eau douce. Dans le cas du poisson marin, considéré dans son biotope, le milieu intérieur est hypotonique par rapport au milieu extérieur qui est hypertonique et l'inverse s'observe chez les poissons d'eau douce. L'isotonie n'est donc pas réalisée et les deux milieux gardent leur concentration.

L'isotonie se heurte alors aux membranes cutanées et branchiales qui ont une action sélective et restrictive.

Cependant, si on plonge ces poissons dans des milieux très différents de leur biotope habituel, des modifications biochimiques apparaissent avec plus ou moins d'intensité et de persistance. Il nous a donc paru intéressant d'étudier dans ces conditions les réactions cardiaques chez les espèces euryhalines et sténohalines.

Nous avons opéré sur des espèces présentant des modes de vie très différents, à savoir, dans l'ordre décroissant d'euryhalinité, l'Anguille (*Anguilla anguilla* Linné), le Muge (*Mugil capito* Cuvier et Valenciennes), le Mulet doré (*Mugil auratus* Risso), la Carpe miroir (*Cyprinus carpio* Linné) et le Griset (*Cantharus cantharus* Cuvier et Valenciennes). Au total, une soixantaine d'individus ont été expérimentés.

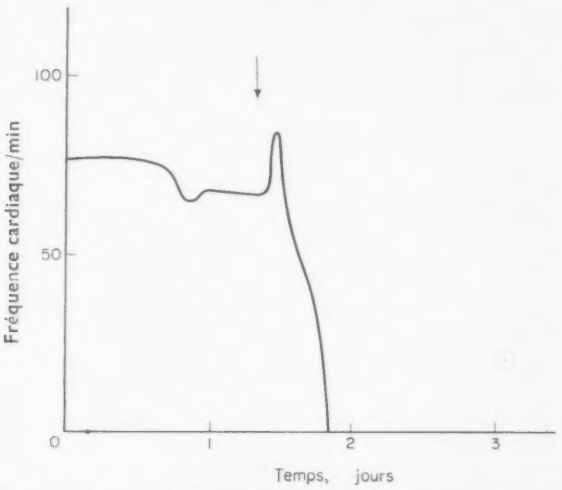

FIG. 1. *Anguilla anguilla* No. 20760 (sans mucosité). La flèche indique le passage du poisson directement de l'eau marine à l'eau douce. Température 22–23°C.

A l'exception de la Carpe conservée en eau douce, puis placée dans une eau salée à 15‰ maximum, tous les individus provenaient du milieu marin, lequel était remplacé très rapidement par de l'eau douce.

Les passages eau de mer–eau douce, ou inversement eau douce–eau salée, ont été réalisés dans l'aquarium même où se trouvaient les individus afin d'éviter tout réflexe aéro-cardiaque dû au contact de l'air. Généralement, le passage en eau douce ou en eau salée s'effectuait en quelques minutes. Les variations de pH, d'oxygénation ou de température ont toujours été très minimes. Au cours de ces changements de salinité, les variations de pH n'ont jamais excédé 0,2 et celles d'oxygène dissout 0,2 ml par litre. Ces modifications sont très insuffisantes pour provoquer des variations chronotropes significatives. Il en est de même en ce qui concerne les variations de température qui, généralement, n'ont pas excédé 1°C; rarement l'écart de température a été de 2°C. Ces variations sont du reste sans influence importante sur les effets étudiés.

Tous les sujets ont été maintenus dans un appareil à contention constitué par un grillage en matière plastique en évitant toute compression des régions céphaliques ou latérales. Un dispositif du type Beaudouin comprenant un électrocardiographe à enregistrement direct sur papier thermo-sensible et un électrocardioscope

permirent de suivre en permanence l'évolution des accidents cardiaques et de fixer les phases intéressantes. Deux électrodes d'un type déjà décrit par l'un de nous (Serfaty & Raynaud, 1956) ont été introduites dans la région précordiale.

Dans le cas de l'Anguille, le passage brutal de l'eau de mer à l'eau douce ne provoque pas de modifications importantes de la fréquence cardiaque. Par contre, l'Anguille marine, débarrassée en partie de son mucus, présente une très forte bradycardie et une apnée importante lorsqu'elle est plongée en eau douce, aboutissant à une mort rapide du sujet (voir Fig. 1).

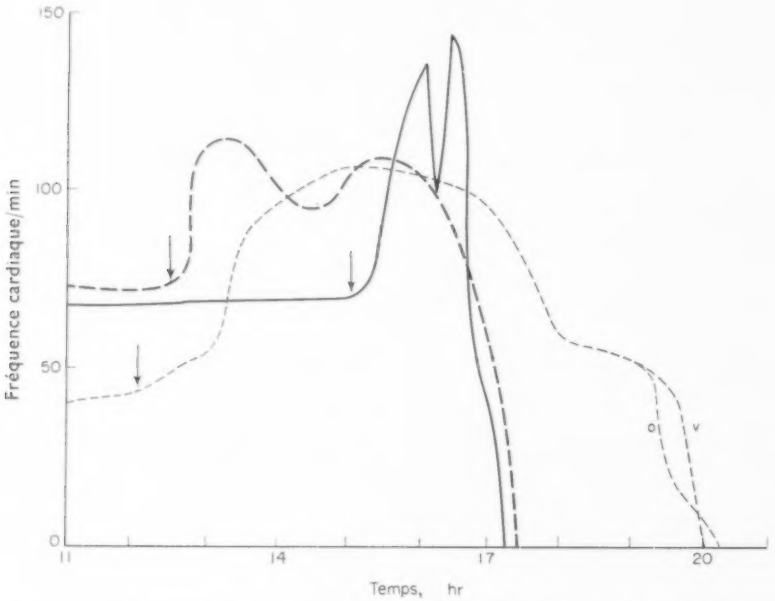

FIG. 2. *Mugil auratus* No. 19859, 24° (— — —); *Mugil capito* No. 20859, 20°C (- - -); *Cantharus cantharus* No. 21859, 22°C (———). La flèche indique le passage du poisson directement de l'eau marine à l'eau douce.

Dans le cas du Muge, chez les deux espèces étudiées (*Mugil capito* Cuiver et Valenciennes, *Mugil auratus* Risso), nous avons observé une résistance de vie d'autant plus importante au passage brutal dans l'eau douce et une tachycardie potamotoque d'autant plus prolongée que les sujets sont plus euryhalins (voir Fig. 2).

Dans le cas du Griset (*Cantharus cantharus*), le passage en eau douce provoque, après une tachycardie précoce, une bradycardie importante déterminant rapidement la mort du sujet (voir Fig. 2).

Dans le cas de la Carpe, les individus supportent aisément des salinités inférieures à 15‰. Lorsque les sujets sont placés dans des salinités n'excédant pas cette concentration, on observe une légère tachycardie tardive mais nette,

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apparaissant seulement 36–48 hr après le passage en eau salée. Leur comportement serait donc voisin de celui des individus euryhalins (voir Fig. 3).

D'une manière générale, il semble que les individus franchement sténohalins comme les Grisets aient une réponse rapide et brutale avec apparition précoce de signes pré-mortels, se manifestant par une tachycardie suivie d'une bradycardie trés importante. Une interprétation de ces variations de fréquence a déjà été

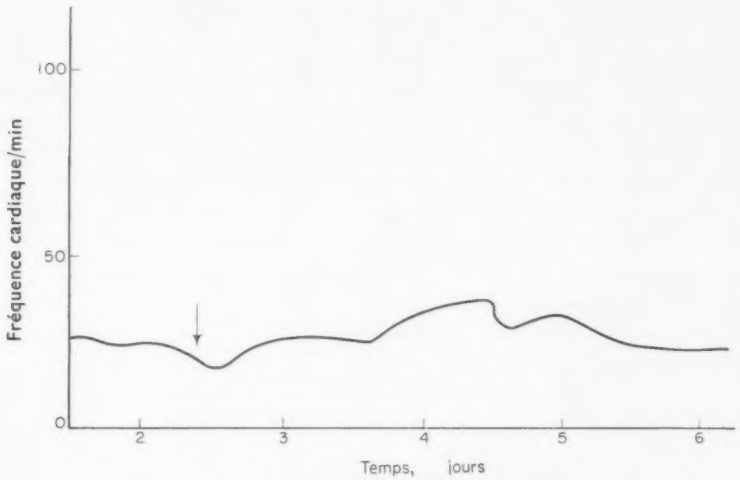

FIG. 3. *Cyprinus carpio* No. 190460. La flèche indique le passage du poisson directement de l'eau douce à l'eau salée à 10,6‰.

donnée récemment par Serfaty & Labat (1960). Ces manifestations font penser à une réaction à dominance nerveuse, comme les mouvements musculaires qui doivent être rapides, mais qui n'ont pas besoin généralement de se maintenir longtemps.

Au contraire, chez les sujets nettement euryhalins comme les Anguilles, les réactions cardiaques sont lentes, peu importantes et durables. Une phase de tachycardie parfois très faible apparaît sans être suivie de bradycardie. Ces mécanismes, qui n'ont pas besoin d'être instantanés, se maintiennent très longtemps. De tels processus font penser à des manifestations à dominance endocrinienne, tels les phénomènes de croissance.

Entre ces deux types extrêmes, se placent tous les cas intermédiaires: *Mugil auratus*, *Mugil capito* notamment.

Ces considérations auraient donc tendance à montrer que les réactions nerveuses sont plus importantes chez les sténohalins que chez les euryhalins.

En effet, l'étude du réflexe aéro-cardiaque (R.A.C.) (Serfaty & Raynaud, 1957) nous a permis d'évaluer l'excitabilité de l'appareil vagal cardiomodérateur et fournit un élément important pour l'appréciation de l'état neuro-végétatif.

Des expériences récentes (résultats inédits) effectuées par M. Waitzenegger et A. Serfaty ont montré que le réflexe aérocardiaque de l'Anguille était moins important notamment que celui des Muges, du Griset ou de la Carpe. Est-ce à dire que le vague des Poissons euryhalins serait moins sensible que celui des individus sténohalins ?

Le cas de l'Anguille démunie du mucus est intéressant à commenter, parce qu'il permet de faire une liaison entre les deux types d'individus.

En effet, l'Anguille intacte présente des réactions euryhalines, l'Anguille débarrassée de son mucus se comporte comme un sténohalin. Tout se passe donc comme si la membrane en absence de mucus devenait perméable aux ions et à l'eau. Une anguille marine ainsi traitée et placée en eau douce présente une bradycardie très précoce et très brutale. Il semble donc que le passage de l'état à dominance hormonale à l'état à dominance nerveuse serait en rapport avec une modification de perméabilité, responsable de troubles hydro-minéraux.

Ces faits seraient en partie vérifiés dans le cas des Carpes. Ces derniers sujets placés dans des solutions salines inférieures à 15‰ se comporteraient en euryhalins, placés dans des solutions de concentration supérieure, le comportement observé est celui d'un sténohalin.

Lors des variations de salinité, il semble que les poissons euryhalins présentent des réactions cardiaques plus lentes et plus durables que les poissons sténohalins chez lesquels des modifications rapides et brutales de la fréquence cardiaque entraînent la mort.

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SHORT COMMUNICATION

AN APPARATUS FOR STUDYING THE GAIN AND LOSS OF ELECTROLYTES BY THE EEL*

D. BELLAMY and I. CHESTER JONES

Department of Zoology, University of Sheffield, England

(*Received* 12 *April* 1961)

Abstract—The apparatus is being used to investigate the effects of hormones on water and electrolyte movements in yellow and silver eels adapted to fresh water and sea water.

EXPERIMENTS on the uptake and excretion of electrolytes by living teleosts require the separation of the fluid in contact with the gills from that surrounding the cloaca. Previous workers have achieved this either by use of a divided chamber with the fish held firmly through the partition, the head in one compartment and the tail in the other (Smith, 1929), or by enclosing the posterior half of the fish in a rubber sac (Meyer, 1951). These methods have the disadvantage of imposing stress on the animal which may mask normal physiological relationships. This short paper presents a new procedure which meets some of the difficulties of experimentation with the eel (*Anguilla anguilla* L.).

It was found that because of the eel's flexible and tubular body, it could be introduced into a glass U-tube so that the head was in one limb with the tail and cloacal opening in the other. When the U-tube was stoppered and inverted, the two limbs held water without the possibility of mixing. The contribution of gills and kidney to the electrolyte balance of the eel could be determined separately by analysing samples of fluid taken from each limb.

The main advantage of the U-tube technique lay in the avoidance of body compression. In addition the small volumes of fluid required rendered ionic changes more readily detectable. The initial results which are included with the description of the apparatus show quite clearly, for the first time, that sodium is taken up from the external medium by the gills of the eel in fresh water to balance that lost in the urine.

The apparatus was essentially a glass tube bent in the form of a U with one limb longer and narrower than the other (Fig. 1). A rubber bung was placed in the open end of the short limb of the inverted U-tube (held in a retort stand) and 70 ml of tap water was introduced through the side tube A. The water was aerated by

* This work forms part of a programme of research in Comparative Endocrinology under the direction of Professor I. Chester Jones, supported by the Agricultural Research Council.

means of a polythene capillary (Pc) passed through tube X. The eel (100 to 150 g wet wt.), held in a dry cloth, was gently pushed into the open end of the long limb (tail compartment) so that its head entered the tap water in the short limb (head compartment). The tip of the tail was bent over, the bung inserted, and the perspex screw plunger (Sp) adjusted until the mouth of the eel almost touched the rubber bung in the head compartment.

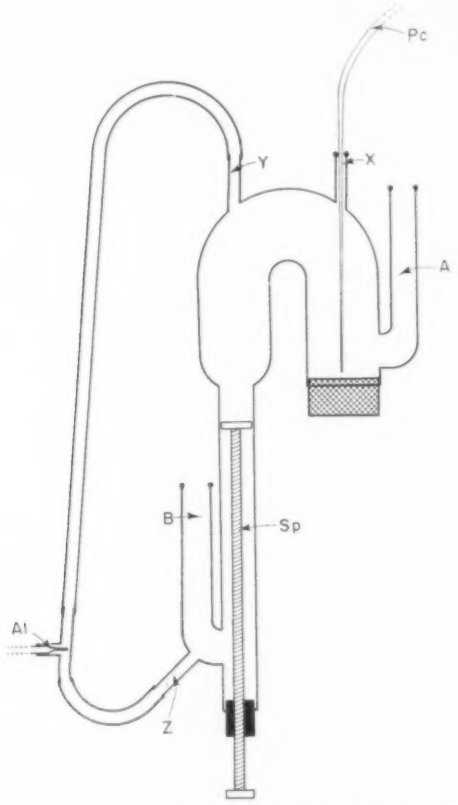

FIG. 1. Apparatus for studying the electrolyte balance of the eel. Scale ⅓ natural size.

An air lift (Al) constructed from polythene tubing was fitted to tube Z and inserted through tube Y until it was positioned over the ventral surface of the eel near the cloaca.

Tap water (80 ml) was then poured into the side tube B. Finally the air lift was adjusted to circulate water over the posterior surface of the eel at a rate of about 1 ml per min.

About 16 hr before the experiment eels were injected intraperitoneally with 0·24 ml of a solution of decamethonium iodide in 0·9% NaCl (0·2 mg/ml) per

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100 g body wt. This was usually sufficient to immobilize the body wall muscle during the period of the experiment (7–48 hr). Movements of the pharynx by which water was drawn into the mouth and expelled through the operculum were not inhibited.

Of several drugs tested decamethonium iodide was the only one which prevented body movements without affecting the respiratory movements. However, there was a great variability in response to the standard dose of 0·24 ml/100 g. Some eels were unaffected by this quantity whilst others were killed within 12 hr. In a few experiments where no drugs were used the results obtained with eels which neither struggled nor produced large amounts of mucus were similar to those with eels immobilized with decamethonium. Thus, the electrolyte movements in the drugged fish did not appear to deviate significantly from those in the normal animal.

RESULTS

The apparatus has been used to measure the rate of uptake and loss of sodium by the silver eel in fresh water. One protocol out of a series of experiments with fasted silver eels (caught during migration to the sea) is presented to show the type of results obtained.

TABLE 1—GAIN AND LOSS OF SODIUM CHLORIDE BY THE FRESH-WATER EEL

Time (hr)	Changes in the sodium chloride content of tap water (μ moles)	
	Head	Tail
1	−6·1	+28·7
2	−15·4	+27·6
4	−22·4	+33·6
7	−30·7	+35·0
14	−39·4	+37·0

A silver eel (114 g wet wt.) was placed in the U-tube with 70 ml tap water in the head compartment (49·4 μ moles Na) and 80 ml tap water surrounding the tail. Samples (1 ml) of each solution were removed at the stated times and the sodium chloride content determined. Temperature 15°C.

With the eel in the apparatus 1 ml samples of the tap water in the head and tail compartments were taken through the side tubes A and B and the sodium chloride content determined (Bellamy, 1961). The results showed that the sodium chloride concentration in the tap water gradually decreased in the head compartment and increased, by a similar amount, in the tail compartment (Table 1). These movements of sodium chloride would be expected if water was taken in osmotically through the gills and excreted as a solution of sodium chloride, the renal sodium

loss being balanced by the active uptake of sodium by the gills (Bellamy, 1961). The latter process, although known in some species of fresh-water teleosts (Krogh, 1939; Meyer, 1951; Wikgren, 1953), has been generally considered not to occur in the eel. The results obtained with the U-tube appear to be the first demonstration of a net sodium uptake by the intact silver eel and confirm previous work with isolated gills.

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A COMPARATIVE STUDY OF NITROGEN EXCRETION IN SOME AMPHIBIA AND REPTILES

MAVIS M. CRAGG, J. B. BALINSKY* and E. BALDWIN

Department of Biochemistry, University College, London

(*Received* 26 *April* 1961)

Abstract—1. The nitrogenous excretion of twelve species of Amphibia and two species of Crocodilia was studied. The urinary content of ammonia, urea, uric acid, and other nitrogenous compounds was determined.

2. Of the Amphibia, completely aquatic forms (all members of the Family Pipidae: *Ambystoma mexicanum*) excrete more ammonia than urea. Partly terrestrial forms excrete mainly urea.

3. The two species of Crocodilia excrete mainly ammonia, some uric acid, and a little urea.

INTRODUCTION

DELAUNAY (1931) first pointed out that the nature of the main nitrogenous waste product in an animal, namely ammonia, urea, or uric acid, is dependent on the environment in which the animal lives. Ammonia, being highly toxic, is the main nitrogenous excretory product only in aquatic animals, which are able to dispose of it rapidly, owing to a large and constant flow of water through them. In terrestrial animals, with a restricted water supply, harmful accumulation of ammonia is prevented by its elaboration into the non-toxic urea or uric acid. As Needham (1931) points out, however, synthesis of urea and uric acid is wasteful both of organic carbon and of energy, and is therefore disadvantageous except where necessitated by water shortage.

Most Amphibia are terrestrial for at least some part of their lives. The work of several authors suggests that they are typically ureotelic. Urea-excreting species include *Rana pipiens* (Van der Heyde, 1921), *R. esculenta* (Przylecki, Opienska, & Giedroyc, 1922), *R. temporaria* (Munro, 1939), *Bufo bufo*, and *Triturus vulgaris* (Munro, 1953). Smith (1929) states that the excretion of *Rana catesbeiana* is very variable, but that ammonia usually predominates. Completely aquatic species, such as *Xenopus laevis* and *Ambystoma mexicanum* (larval), appear to be ammoniotelic (Munro, 1953).

Among the Reptiles, the Squamata are uricotelic (Needham, 1931). The Chelonia have been investigated by Moyle (1949). She finds an interesting correlation of nitrogen excretion and environment. Aquatic species excrete approximately equal amounts of ammonia and urea. Terrestrial forms are ureotelic if preferring damp surroundings, and uricotelic if living in dry localities. Among the Crocodilia,

* Present address: Division of Biochemistry, Department of Botany, University of the Witwatersrand, Johannesburg, South Africa.

Alligator mississippiensis is ammoniotelic (Hopping, 1923; Coulson, Hernandez & Brazda, 1950). Kahlil & Haggag (1958) found that *Crocodylus niloticus* excretes its waste nitrogen partly as a fluid, containing predominantly ammonia, and partly as a solid deposit, containing mainly uric acid. Their approximate calculations suggest that in the over-all excretion uric acid predominates.

It was considered desirable to carry out a more comprehensive investigation of a large number of species of Amphibia, in an attempt to correlate nitrogen excretion with environment. Twelve species of Anura and Urodela, living in

TABLE 1—EXPERIMENTAL ANIMALS AND THEIR HABITATS

Species	Habitat (outside the breeding season)
Cl. AMPHIBIA	
Ord. URODELA	
Salamandra salamandra	terrestrial
Triturus cristatus	terrestrial; can live in water
Ambystoma mexicanum (neotenic larva)	aquatic
Ord. ANURA	
Fam. Pipidae	
Xenopus laevis	aquatic
Xenopus tropicalis	aquatic
Hymenochirus sp. (*boettgeri* ?)	aquatic
Pipa pipa	aquatic
Fam. Ranidae	
Rana esculenta	semi-aquatic
Rana temporaria	terrestrial
Fam. Hylidae	
Hyla arborea	terrestrial, living in trees
Fam. Bufonidae	
Bufo bufo	terrestrial, preferring dry environment
Bufo calamita	terrestrial, preferring dry environment
Cl. REPTILIA, Ord. CROCODILIA	
Crocodylus niloticus	aquatic, but able to leave water
Caiman crocodilus	aquatic, but able to leave water

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various habitats, from dry terrestrial to completely aquatic, were chosen for investigation. Special attention was paid to species which have reverted to an aquatic mode of life, particularly the Family Pipidae, which includes *Xenopus*, in order to ascertain to what extent they have returned to ammonia excretion. Two species of Crocodilia have also been investigated.*

MATERIALS AND METHODS

Maintenance of animals. The species investigated, together with their natural habitats, are shown in Table 1. All Amphibia were kept in glass aquaria. *Ambystoma*

* Experiments on the terrestrial Amphibia and Crocodilia were carried out by M.M.C. and E.B. (Cragg, 1953). The Pipidae were subsequently investigated by J.B.B. and E.B.

mexicanum, *Triturus cristatus*, and the Pipidae were kept in water about 6 in. deep. The *Rana* species and *Hyla arborea* were kept in one inch of water, with stones and twigs projecting above the water level. *Salamandra salamandra* and the two *Bufo* species were kept on damp sand.

The *Xenopus* species and *Ambystoma* were fed twice a week on chopped liver or heart. *Hymenochirus* were fed on *Tubifex* placed in their tank twice a week. The other species of Amphibia were all fed on earthworms and blowflies three times a week.

The crocodiles were not kept in the laboratory, their urine samples being collected in the Reptile House of the Zoological Society of London.

Collection of urine from Amphibia. Before each experiment, the animals' bladders were emptied. Urination occurred on handling, or on massaging the abdomen. The animals were then washed, dried, and weighed. 24-hr urine samples were collected by one of the methods described below. Usually, several conspecific animals were used at once, and their urines were pooled.

Method 1, for semi-terrestrial Amphibia. The animals were placed in a crystallizing dish provided with a glass tube at the bottom, through which the urine drained into a centrifuge tube. The tube contained a drop of dilute sulphuric acid, to prevent loss of ammonia and bacterial decomposition of urea. The crystallizing dish was covered with a tightly fixed perforated sheet of paper. The dish and centrifuge tube were covered by a bell jar. Air was drawn into the bell jar through dilute sulphuric acid, and out through a 2 per cent solution of boric acid, to trap any ammonia which had volatilized. The atmosphere inside the bell jar was kept moist by the presence of pieces of wet cotton wool. After 24 hr, the animals' bladders were again emptied, and both the dish and the animals were washed, the washings draining into the centrifuge tube. The urine and washings were centrifuged to remove any solid material, and made up to a suitable volume.

Method 2, for aquatic Amphibia (*Ambystoma*, *Triturus*, and the Pipidae). The animals were kept in a covered dish containing just sufficient water to cover them. A few drops of a 0·02 per cent solution of terramycin (intramuscular, Chas. Pfizer & Co., Inc., New York) were added to the water, to prevent bacterial decomposition of urea (Balinsky & Baldwin, 1961). Originally the container was kept under the bell jar, but as no ammonia was collected in the boric acid trap, this procedure was later omitted. After 24 hr, the animals and the surrounding fluid were poured into a Büchner funnel, the fluid draining into a container beneath. The animals were washed in the funnel. The fluid thus collected was centrifuged, brought to pH 1–2 with 10 N sulphuric acid, and made up to a suitable volume.

Both methods were used for some excretion measurements in *Xenopus laevis*, *Triturus cristatus*, *Salamandra salamandra*, and *Bufo calamita*, and yielded similar results. The results obtained by the two methods are, therefore, comparable.

Collection of urine from Crocodilia. One animal at a time was placed in a perspex cylinder with a perforated brass plate screwed on at each end. The cylinder was fixed at an angle of about 20° to the horizontal. A baffle of perspex was inserted about an inch from the lower end, with an outlet tube just above it. The urine thus

drained along the side of the cylinder, was stopped by the baffle, and flowed via the drainage tube into a small flask containing toluene.

As noted by Khalil and Haggag (1958), the urine sample consists of a colourless liquid and a solid white deposit. It was diluted with distilled water, adjusted to pH 7–8 with dilute hydrochloric acid, and brought to the boil, all the white solid material going into solution. The fluid was filtered, and made up to a suitable volume.

Aliquots of the urine samples collected by the above methods were taken for assay of ammonia, urea, amino acids, uric acid, creatinine, creatine, allantoin, and total non-protein nitrogen.

Analytical methods

Ammonia nitrogen was determined by distillation in Markham's (1943) apparatus, the distillate being collected in a 2 per cent solution of boric acid containing the indicator of Conway & O'Malley (1942). In later experiments, Russell's (1944) colorimetric method was used, with modified reagents (Balinsky & Baldwin, 1961).

Urea nitrogen was estimated by hydrolysing to ammonia with urease, contained in an extract of soya bean meal (Krebs & Henseleit, 1932). The ammonia nitrogen was measured as described above. Urea nitrogen was given by the difference between the ammonia nitrogen before and after treatment with soya bean extract. A blank determination with soya bean extract alone was also carried out, and the result subtracted from the experimentally determined amount of urea nitrogen. In later experiments, the colorimetric method of Archibald (1945), with slightly modified reagents (Balinsky & Baldwin, 1961), was used.

Amino acid nitrogen was converted to ammonia by treatment with ninhydrin (Sobel, Hirschman & Besman, 1945) and calculated from the difference between ammonia content before and after treatment with ninhydrin. Urea also yields some ammonia by hydrolysis under these conditions. The amount of hydrolysis was determined using standard amounts of urea. Knowing the amount of urea present in the sample, the appropriate correction was applied to the amino acid determination.

Uric acid was measured by the method of Dresel & Moyle (1950).

Creatinine was determined by Folin's picrate method as described by Borsook (1935).

Creatine was converted to creatinine by boiling with picric acid (Cole, 1953), and determined as creatinine.

Allantoin was estimated by the method of Young & Conway (1942).

Total non-protein nitrogen. Protein was precipitated from the urine sample (8 volumes) with 10 per cent sodium tungstate (1 volume) and 0·7 N sulphuric acid (1 volume). The total non-protein nitrogen was estimated by digesting for 6 hours with 2 ml of concentrated sulphuric acid and the catalyst of Chibnall, Rees & Williams (1943). The ammonia was then estimated in Markham's apparatus as described above.

RESULTS

Amphibia

No ammonia was collected in the boric acid traps. This is in agreement with the findings of Przylecki *et al.* (1922), indicating that no ammonia is lost to the atmosphere through the skin or lungs of Amphibia.

The results of the ammonia, urea, and total non-protein nitrogen measurements are shown in Table 2. It will be seen that urea is the predominant nitrogenous

TABLE 2—PARTITION OF NITROGEN EXCRETED BY TWELVE SPECIES OF AMPHIBIA

The figures quoted are mean values, followed by standard deviations in brackets.

Species	No. of experiments	Total non-protein N (μg/g body wt./24 hr)	Urea N (μg/g body wt./24 hr)	Ammonia N (μg/g body wt./24 hr)	Ammonia N as percentage of ammonia N + urea N	
					Mean	Standard error of mean, $\pm$
Salamandra salamandra	5	76·6 (30·7)	62·7 (20·2)	3·4 (2·6)	4·70 (3·23)	1·44
Triturus cristatus	6	84·5 (49·1)	67·3 (42·1)	2·8 (1·9)	4·05 (1·87)	1·65
Ambystoma mexicanum	8	174·8 (55·9)	49·4 (21·1)	74·3(30·6)	61·94 (9·94)	3·51
Xenopus laevis	82	—	107·3 (77·9)	165·2(68·0)	62·23(11·95)	2·63
Xenopus tropicalis	6	—	80·2 (33·8)	128·2(36·3)	61·67(15·74)	6·43
Hymenochirus sp.	6	—	28·5 (19·5)	96·2(15·3)	78·03(12·61)	5·15
Pipa pipa	3	—	7·5 (1·6)	101·3(40·7)	92·53 (2·78)	1·66
Rana esculenta	7	63·6 (23·9)	48·9 (21·8)	4·5 (1·5)	9·43 (3·75)	1·42
Rana temporaria	6	118·7 (60·4)	92·7 (44·6)	8·8 (5·7)	8·24 (2·10)	0·86
Hyla arborea	8	174·3 (54·3)	149·9 (49·8)	7·1 (3·3)	4·60 (1·40)	0·50
Bufo bufo	7	117·6(111·2)	106·3(102·2)	6·2 (3·4)	4·77 (1·25)	0·47
Bufo calamita	9	98·9 (62·1)	85·7 (53·9)	5·8 (5·7)	5·72 (2·31)	0·77

waste product in *Salamandra salamandra*, *Triturus cristatus*, *Rana esculenta*, *R. temporaria*, *Hyla arborea*, *Bufo bufo*, and *B. calamita*. The percentage of ammonia in the Ranidae is higher than that of the other ureotelic Anura. The difference in all cases is significant at the 5 per cent level.

Ambystoma mexicanum and all the Pipidae investigated are ammoniotelic. The urea excretion of *Xenopus* species is still considerable. *Pipa pipa* and *Hymenochirus* sp. excrete the least amount of urea. In general, the percentages of ammonia are lower than those obtained by Munro (1939, 1953). It is interesting to note the high values of standard deviation in the percentage of urea in the excretion of *Xenopus*, *Hymenochirus*, and *Ambystoma mexicanum*. It seems that great variability exists in the relative amounts of the two waste products in these species. A similar variability was observed in *Ambystoma mexicanum* by Munro (1953), and in the lungfish *Protopterus aethiopicus* by Smith (1930). It appears to occur whenever the two waste products are excreted in comparable amounts.

The results of determinations of other nitrogenous waste products are shown in Table 3. It will be seen that, though amino acids are excreted by some of the species in significant amounts, they do not appear to form a major nitrogenous waste product in Amphibia. Uric acid, creatine, and creatinine are all excreted in very small amounts. Incomplete conversion of creatine to creatinine is indicated by the fact that there is always more of the former than of the latter. Both compounds are present, however, in all species investigated. Zwartenstein (1929) was able to

TABLE 3—MINOR NITROGENOUS WASTE PRODUCTS OF AMPHIBIA

Species	Mean percentage of total non-protein nitrogen excreted as			
	Amino acids	Uric acid	Creatinine	Creatine
Salamandra salamandra	0·0	—	—	—
Triturus cristatus	0·3	0·2	0·5	1·3
Ambystoma mexicanum	3·8	—	—	—
Xenopus laevis	2·8	0·15	0·2	0·3
Rana esculenta	5·8	0·05	—	—
Rana temporaria	3·8	0·1	—	—
Hyla arborea	0·0	0·25	—	—
Bufo bufo	0·1	—	—	—
Bufo calamita	0·6	0·45	0·2	0·5

detect only creatine in the urine of *Xenopus laevis*. After allowing for the colour produced in the determination by uric acid, no excretion of allantoin could be detected. This is in agreement with the presence of allantoinase in Amphibia (Brunel, 1937).

TABLE 4—PARTITION OF NITROGEN EXCRETED BY TWO SPECIES OF CROCODILIA

The figures quoted are mean values, followed by the standard errors of the means, with standard deviations in brackets underneath.

Species	No. of expts.	Percentage of total non-protein nitrogen excreted as		
		Ammonia	Uric acid	Urea
Crocodylus niloticus	7	66·2 ± 3·49 (9·24)	20·8 ± 3·47 (9·18)	4·5 ± 1·06 (2·80)
Caiman crocodilus	6	52·5 ± 5·21 (12·74)	27·3 ± 5·88 (14·41)	5·9 ± 1·30 (3·18)

Crocodilia

The urine of *Crocodylus niloticus* contained 1·80–2·25 mg of non-protein nitrogen per millilitre, and that of *Caiman crocodilus* 1·34–2·14 mg. Table 4 shows the partition of nitrogen in the two species. Ammonia is the most important waste product, followed by uric acid and then by urea. The relative amounts of ammonia and uric acid show considerable variability.

The urine of both species has a pH of 8–9. No amino acid nitrogen could be detected. A few estimations of other non-protein constituents were made. Creatine and creatinine nitrogen varies from 0·5 to 3 per cent of the total, the former being the larger fraction. The allantoin nitrogen is 1–2 per cent of the total in *Crocodylus*, and 3–4 per cent in *Caiman*.

DISCUSSION

The object of the present investigation was to determine to what extent the nitrogenous waste products are correlated with the environment of each species. The results certainly emphasize the importance of the environment. Among the Amphibia, which are generally mainly terrestrial and ureotelic, completely aquatic forms are ammoniotelic. Ammoniotelism is found in the Pipidae among the Anura, and in *Ambystoma mexicanum* among the Urodela.

On morphological grounds, it is assumed that the Pipidae are derived from more terrestrial ancestors, and hence it appears that ammoniotelism is a secondary acquisition, probably evolved soon after the return to water of the primitive Pipidae. In the neotenic *Ambystoma mexicanum*, ammoniotelism is merely a larval character, retained by the adult because it does not normally undergo metamorphosis. When metamorphosis is induced, ureotelism also results (Munro, 1953).

It seems probable that ammonia excretion has adaptive value to these animals. The most likely explanation of this is that of Needham (1931), namely that urea synthesis constitutes a waste of carbon and energy. Another possible advantage of ammoniotelism is that it aids the retention of univalent cations by exchange of these for ammonium ion in the kidney tubules (Coulson *et al.*, 1950).

It should be noted that ammoniotelism is restricted to completely aquatic Amphibia. Species such as *Rana esculenta* and *Triturus cristatus*, which may spend much of their time in water even outside the breeding season, produce mainly urea. In other words, in Amphibia which live partly in and partly out of water, nitrogen excretion is fitted for the terrestrial mode of life. Ureotelic species kept in water (urine collected by Method 2) continue to excrete urea in aquatic surroundings. It appears, therefore, that the nature of the predominant waste product in these species is determined by adaptations of enzymic mechanisms rather than by the direct effect of the environment.

A relatively greater percentage of ammonia is excreted by the *Rana* species. While *Rana temporaria* is considerably less aquatic than *R. esculenta*, it might be significant that the Genus *Rana* is, in general, much more dependent on water than, say, *Bufo*, and much less likely to be exposed to serious dehydration. A less complete conversion of ammonia to urea than is necessary in *Bufo* might thus be tolerated in *Rana*. It is also interesting that, among the Pipidae, the smallest amount of urea is excreted by the most specialized forms, *Hymenochirus* and *Pipa*.

The present results for *Crocodylus niloticus* and *Caiman crocodilus* suggest that, in keeping with their predominantly aquatic habitat, both species are ammoniotelic, resembling in this *Alligator mississippiensis* (Hopping, 1923; Coulson *et al.*, 1950).

The approximate calculations of Khalil & Haggag (1958) suggest a higher percentage of uric acid in the excreta of *Crocodylus niloticus* than indicated by the present experiments. As these authors observe that the ratio of liquid urine to solid deposit appears to be very variable, differences in the percentage of uric acid might well be reflections of varying relative amounts of solid and liquid excreted. Differences of this kind might well be conditioned by the environment in which the experimental animals were kept. Unfortunately, it is not clear under what conditions the experimental animals of Khalil & Haggag were living. The specimens used in the present investigation were taken from the terraria of the Reptile House, where they were able to spend some of their time submerged in water.

The present results are in agreement with Delaunay's (1931) generalization that aquatic animals are ammoniotelic. This generalization appears to hold even for species which belong to predominantly ureotelic or uricotelic groups. Secondarily aquatic forms appear to have reverted to ammoniotelism when they abandoned their terrestrial mode of life.

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THE ADAPTATION OF AMPHIBIAN WASTE NITROGEN EXCRETION TO DEHYDRATION

J. B. BALINSKY,* MAVIS M. CRAGG and E. BALDWIN

Department of Biochemistry, University College, London

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Abstract—1. Specimens of *Xenopus laevis*, when kept out of water for 1–3 weeks, excreted little nitrogen. On return to water, large amounts of urea, but only normal amounts of ammonia were excreted.

2. Accumulation of urea in blood, liver and muscle during dehydration was demonstrated.

3. Urea accumulation could also be induced by keeping the animals in 0·9 per cent sodium chloride.

4. *Bufo calamita* kept in a dry tank also accumulated urea.

5. The rate of excretion of accumulated urea in *Xenopus* appeared to follow an exponential plot, indicating passive excretion. In *Bufo calamita*, the excretion plot was not exponential.

6. The adaptive significance of urea accumulation during dehydration is discussed. In *Xenopus*, urea represents an "emergency" waste product, replacing ammonia in dry conditions.

7. A theory of the evolution of ureotelism in Amphibia is suggested.

INTRODUCTION

In our previous paper (Cragg, Balinsky, & Baldwin, 1961), describing a study of the nitrogen excretion of a number of species of Amphibia, it was shown that the nature of the nitrogenous waste product is closely correlated with the animal's habitat. Fully aquatic species are ammoniotelic, while those which spend at least part of their time on land excrete predominantly urea. In the course of amphibian development, too, the nature of the waste product is fitted to the environment, being mainly ammonia in the aquatic larva, and urea in the terrestrial adult (Munro, 1939, 1953).

It was considered interesting to observe the effect of changing the environment on the nitrogen excretion of adult Amphibia. An example of such an effect has been described in the African lungfish, *Protopterus aethiopicus* by Smith (1930). This animal was found to excrete more ammonia than urea when living in water. When aestivating in the mud, however, no nitrogen was excreted, but urea accumulated in the tissues, reaching a concentration of some 10 mg nitrogen/g after a year of aestivation. On reimmersion in water, very large quantities of urea were excreted, but only normal amounts of ammonia.

Among the Amphibia, aestivation and hibernation under dry conditions is often encountered. *Bufo calamita* is known to spend long periods of time in burrows in

* Present address: Division of Biochemistry, Department of Botany, University of the Witwatersrand, Johannesburg, South Africa.

relatively dry soil (Werner, 1912). The completely aquatic *Xenopus laevis*, according to Rose (1950), is often found in pools freshly formed after rain, indicating that the animals had previously been aestivating in the mud. That *Xenopus* can undertake land journeys is indicated by the finding of dead specimens on the roads.

An attempt has been made in the present investigation to simulate artificially the dry environment of these animals, and to observe its effect on their nitrogen excretion.*

A preliminary report of some of the results has already been published (Balinsky, 1960).

MATERIALS AND METHODS

Experimental animals and urine collection

The species investigated were *Xenopus laevis* and *Bufo calamita*. The maintenance of the animals and the collection of urine from them are described in a previous paper (Cragg *et al.*, 1961). Method 1 was used when the animals were kept out of water, and Method 2 when they were in water.

Analytical methods

Urine analysis for ammonia, urea, amino acids, and total non-protein nitrogen was carried out as described by Cragg *et al.* (1961). Urine volume was determined as described by Balinsky & Baldwin (1961a).

Tissue and blood urea. Pieces of liver and leg muscle were removed from freshly killed animals, and homogenized with 10 to 20 times their volume of distilled water in an M.S.E. top-drive homogenizer. The homogenates were deproteinized with a third of their volume of 20 per cent trichloroacetic acid, centrifuged to remove the precipitate, and made up to a suitable volume. Aliquots were analysed for urea by Archibald's (1945) colorimetric method. The slightly modified reagents used for this determination, as well as the method used for blood urea determinations are described by Balinsky & Baldwin (1961a).

Arginase activity was determined as described by Balinsky & Baldwin (1961b).

RESULTS

Xenopus laevis

Several specimens of *Xenopus laevis* were kept in a glass tank containing damp grass but no water. They were sprinkled with water periodically and fed as usual. The animals were weighed before the commencement of the experiment and at intervals throughout its duration. A weight loss due to evaporation of up to 24 per cent of the body weight was observed. The animals were then reimmersed in water, when their body weight returned to the original value. Measurements of nitrogen excretion were made during the period of desiccation, and for several days after return to water. The results are shown in Table 1.

* The first experiments were carried out by M.M.C. and E.B. (Cragg, 1953), the work being continued by J.B.B. and E.B.

It will be seen that, while out of water, *Xenopus* excreted only small amounts of waste nitrogen, the percentages of ammonia and urea not being different from normal. On return to water, large amounts of nitrogenous waste products were voided, which must evidently have been stored in the body during desiccation.

TABLE 1—NITROGEN EXCRETION OF *Xenopus laevis* KEPT OUT OF WATER AND ON RETURN TO WATER

Environment	*Expt. No.* 1 (19 days out of water)				*Expt. No.* 2 (7 days out of water)			
	Total non-protein N (μg/g body wt./24 hr)	Urea N (μg/g body wt./24 hr)	Ammonia N (μg/g body wt./24 hr)	Ammonia N as percentage of ammonia N + urea N	Total non-protein N (μg/g body wt./24 hr)	Urea N (μg/g body wt./24 hr)	Ammonia N (μg/g body wt./24 hr)	Ammonia N as percentage of ammonia N + urea N
Living in water	—	—	—	—	*206·9	*66·3	*122·3	*66·3
Out of water (on damp grass)	*43·8	*15·4	*20·9	*57·6	—	—	—	—
Returned to water, day after return:								
1 (first 6 hours)	—	—	—	—	†56	†18	†31	63·3
(remaining 18 hours)	—	—	—	—	‡685	‡537	‡124	18·8
(total 24 hours)	—	—	—	—	741	555	155	22·0
2	—	—	—	—	704	466	173	27·1
3	505	382	85	18·2	436	228	188	45·2
5	314	205	70	25·4	—	—	—	—
6	—	—	—	—	243	112	112	50·0
7	—	—	—	—	215	109	92	44·5
10	170	59	96	62·0	113	62	46	42·5

* means of 6 expts. † excretion per 6 hr. ‡ excretion per 18 hr.

The large nitrogen output on return to water was almost entirely due to increased excretion of urea, the output of ammonia being approximately normal. Fig. 1 shows the plot of the logarithms of urea excretion on successive days after the return to water. It will be seen that the two plots are very similar and appear to be linear, indicating that urea excretion fell off exponentially.

Table 1 also shows that there was an initial lag of a few hours in the urea output after return to water. This probably represents the period before the water lost by evaporation had been fully regained.

The level of amino acid excretion after dehydration was not significantly higher than the normal level (Cragg *et al.*, 1961). The volume of urine produced on the first day after return to water was also very similar to the normal urine output of *Xenopus* living in water (Balinsky & Baldwin, 1961a).

The results of blood and tissue urea measurements in the animals which had been kept out of water are shown in Table 2, and compared to the corresponding

values for those living in water. It will be seen that there was a very large increase of urea content in all tissues, indicating that the urea was stored in all parts of the body.

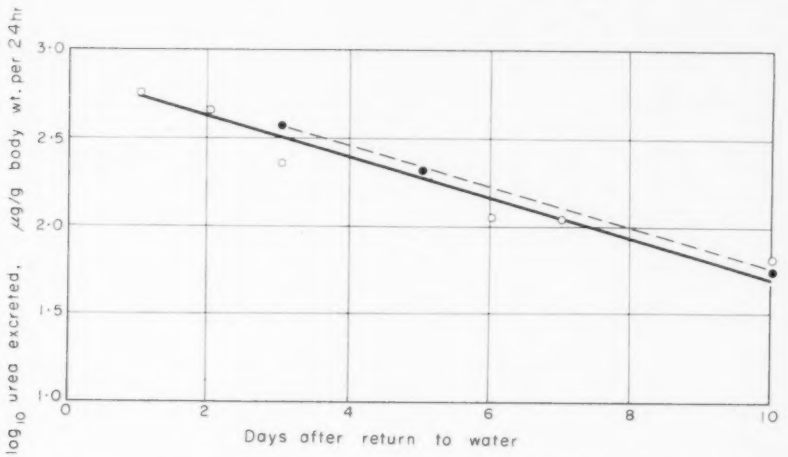

FIG. 1. Urea excretion of *Xenopus laevis* on return to water after being kept out of water (on damp grass). ●—● Expt. No. 1 (19 days out of water). ○—○ Expt. No. 2 (7 days out of water). (Urea on logarithmic scale.)

TABLE 2—BLOOD AND TISSUE UREA CONCENTRATION IN *Xenopus laevis* KEPT IN AND OUT OF WATER

The figures quoted are mean values, followed by standard deviations in brackets.

Environment	No. of expts.	Urea concentration, in µg nitrogen per g wet wt.		
		Blood	Liver	Muscle
Living in water	15	172 (122·9)	—	—
	4	—	202 (62·1)	160 (82·8)
Kept out of water, on grass for 14 days	1	1420	960	920
	1	1360	1230	830
	1	2410	—	—
Kept out of water, on wet peat for 10 days	4	—	540 (46·9)	562 (56·6)

In order to determine whether water shortage or some other factor is responsible for the accumulation of urea, two experiments were carried out, the results of which are shown in Table 3. In the first, several animals were kept out of water, but allowed to burrow in wet peat. No loss of weight by dehydration occurred. After

10 days, they were returned to water, and their excretion was measured. Table 3 indicates that the amount of urea excreted was not nearly as great as in dehydration experiments, though a higher percentage of urea than usual was excreted. Measurements of tissue urea were also made, and are shown in Table 2. The urea content was higher than normal, but far below the level observed in experiments where loss of weight by dehydration could be observed.

Table 3—Nitrogen excretion of *Xenopus laevis* kept (a) on wet peat, (b) in hypertonic solution, and on return to distilled water

The figures quoted are mean values, followed by standard deviations in brackets.

Environment	No. of expts.	Urea N (μg/g body wt./ 24 hr)	Ammonia N (μg/g body wt./ 24 hr)	Ammonia N as percentage of ammonia N + urea N
First day in dist. water, after being kept on wet peat for 10 days	5	105·8 (61·9)	85·2 (15·3)	47·7 (12·9)
Seventh day in 0·9% sodium chloride	4	93·0 (32·7)	72·8 (12·1)	45·3 (10·7)
First day on return from 0·9% sodium chloride to dist. water	4	289·3 (48·8)	52·8 (21·3)	15·2 (4·1)

In the second experiment, the animals were kept in a 0·9 per cent solution of sodium chloride (a solution hypertonic to frogs) for six days. The excretion on the last day in salt water and on the first day in distilled water was measured, and is shown in Table 3. It will be seen that, on return to distilled water, a large increase in urea excretion occurred, very similar to that observed in the dehydration experiments. The only factor common to the two types of experiment is water shortage, induced in the one case by evaporation, and in the other by a hypertonic environment. It can be concluded that urea accumulation is caused by water shortage, and the extent of urea accumulation in the tissues is dependent on the degree of water shortage.

The liver arginase activity of two animals subjected to dehydration was measured, and found not to differ from the normal values for the species (Balinsky & Baldwin, 1961b).

Bufo calamita

Several specimens were kept in a dry tank with grass and leaves for fourteen days, and fed as usual. On the first two days, a few drops of urine were voided, but after that there was no further production of urine. By the eighth day, a mean weight loss of 12·3 per cent was observed, after which the weight remained constant. From the fifteenth day onwards, the animals were partially immersed in

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water, and their subsequent nitrogenous excretion was measured. The results are shown in Table 4. It will be seen that, as in *Xenopus*, there was a very large output of waste nitrogen on the first day in water, followed by a gradual drop of the

TABLE 4—NITROGEN EXCRETION OF *Bufo calamita* AFTER BEING KEPT DRY IN A TANK FOR 14 DAYS

Day after return to water	Total non-protein N (μg/g body wt./ 24 hr)	Urea N (μg/g body wt./ 24 hr)	Ammonia N	
			(μg/g body wt./ 24 hr)	as percentage of ammonia N + urea N
1	1080	880	98	10·0
2	530	451	31	6·4
3	382	329	19	5·5
4	397	347	19	5·2
8	318	278	17	5·8

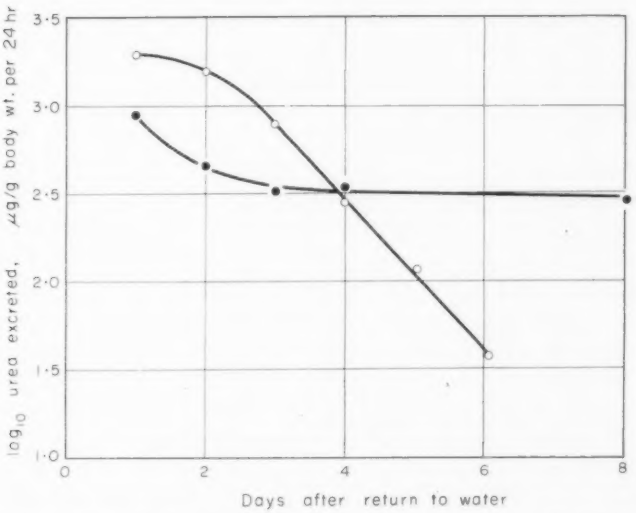

FIG. 2. Urea excretion of *Bufo calamita* and *Protopterus aethiopicus* on return to water after being kept out of water. ●—● *Bufo calamita*, previously kept in a dry tank for 14 days. ○—○ *Protopterus aethiopicus*, previously kept in mud for a year. Data after Smith (1930, Protocol No. 1). (Urea on logarithmic scale.)

output. Here, too, excretion of stored waste nitrogen had evidently occurred. This time, however, an increase in ammonia as well as urea output was observed, so that the percentage of ammonia remained the same. Moreover, the logarithmic plot of urea output (Fig. 2) in *B. calamita* is not linear.

DISCUSSION

The present results indicate that, when Amphibia are subjected to water restriction, and produce little urine, they store waste nitrogen in their bodies. This nitrogen is stored in the form of urea, irrespective of whether the animal is normally ureotelic or not. The animals are thus able to tolerate blood and tissue urea concentrations many times greater than normally occur in them. This is exactly analogous to the behaviour of *Protopterus aethiopicus* (Smith, 1930). It should be noted, however, that the tissue urea concentrations observed in aestivating *Protopterus* and also in Elasmobranchs are still four times greater than those in the present experiments.

Xenopus laevis further resembles *Protopterus* in that, although it normally excretes large amounts of ammonia, it is apparently able to channel the entire waste nitrogen into urea under conditions of water shortage.

The more drastic the dehydration, the greater is the accumulation of urea. It should be borne in mind that water restriction does not necessarily imply actual weight loss by dehydration. Restricted water intake may to some extent be balanced by reduced urine production, resulting in reduced water turnover without loss of weight. The results shown in Tables 2 and 3 suggest that, under such conditions, some urea accumulation does take place, but to a much smaller extent.

The fact that, when out of water, *Xenopus* excretes ammonia and urea in small amounts, but in the same proportions as usual, suggests that decreased waste nitrogen excretion is merely a reflection of the small volume of urine produced. In other words, a reduction of urine flow in response to dehydration automatically reduces the amount of waste nitrogen excreted.

It is significant that only urea is accumulated in the tissues. This is of great advantage to the animal, as any accumulation of ammonia would be highly toxic. The mechanism of accumulation of urea only may perhaps be understood if one considers the sites of formation of the two waste products. Urea synthesis occurs in the liver of *Amphibia* (Manderscheid, 1933), and is thus probably independent of urine flow. Ammonia formation appears to take place in the kidney of *Xenopus* (Balinsky & Baldwin, 1961a, b), and might be profoundly affected by the process of water regulation. Thus a restricted urine production might curtail the formation as well as the excretion of ammonia, but only the excretion of urea. This would lead to an accumulation of urea in the body.

It has already been pointed out (Cragg *et al.*, 1961; Balinsky & Baldwin, 1961a) that *Xenopus* does synthesize considerable amounts of urea when living in water, and that it must therefore always possess at least small amounts of the enzymes necessary for urea synthesis. The conversion of the entire waste nitrogen into urea, however, implies that urea-producing activity is above the usual level. The stimulation of this process may be merely the result of mass action, through the accumulation of some precursors which are normally converted to ammonia. The possibility of a stimulation of activity of urea cycle enzymes can, however, not be ruled out. No such stimulation could be observed in the case of arginase.

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It is interesting to note the difference in the rate at which accumulated urea is excreted in *Xenopus laevis* and in *Bufo calamita* (Figs. 1 and 2). The exponential nature of the *Xenopus* curve indicates that urea excretion is proportional to the concentration of urea in the body. Such a relationship would hold if the excretion occurred entirely by passive glomerular filtration. The results of Balinsky & Baldwin (1961a) indicate that urea is passively filtered by *Xenopus*, and excretion of accumulated urea is thus merely a special case of this more general phenomenon. The non-exponential curve of *Bufo calamita* could be due to the participation of active tubular secretion as well as passive filtration in the process of urea excretion.

It is interesting to note that the excretion of urea by *Protopterus* after aestivation resembles that of *Xenopus*. The results of one of Smith's (1930) experiments (Protocol 1) are plotted in Fig. 2, on the same logarithmic scale as the present results. It will be noted that, just as in *Xenopus*, there is an initial lag, followed by exponential excretion of urea. It appears that in *Protopterus*, too, excretion of accumulated urea is passive.

It seems likely that storage of urea in the tissues sometimes occurs under natural conditions, when the water turnover in the animals is unusually low. This could occur either during land journeys by aquatic species, or during aestivation or hibernation. Even water restriction which is less severe than in the present experiments might, over a long period of time, lead to considerable urea accumulation. Though aestivating or hibernating animals do not feed, it should be borne in mind that starving Amphibia metabolize mainly protein (Przylecki & Karczewski, 1923), and must therefore continue to form nitrogenous waste products.

It seems likely that the ability to tolerate considerable amounts of urea in blood and tissues plays an important role in enabling Amphibia to survive under the conditions described. *Xenopus laevis* is of special interest, as urea is not the main nitrogenous waste product in aqueous habitat. It can rather be regarded as an "emergency" waste product, which is normally secondary, but comes into play when waste nitrogen has to be stored, enabling the animal to survive dehydration.

The possession of an "emergency" nitrogenous waste product by animals such as *Xenopus* or *Protopterus* carries with it the advantage of adaptability to either aquatic or terrestrial environment. Ureotelism appears to be disadvantageous in aquatic surroundings (Cragg *et al.*, 1961), and is rarely found in aquatic animals. *Xenopus* and *Protopterus* combine ammoniotelism with the possession of the mechanism of ureogenesis. This condition gives the ability to survive drought while it does not carry with it the disadvantages of ureotelism. It is thus well adapted to the animals' way of life.

The power of making an "emergency" nitrogenous waste product may well be possessed by many aquatic animals which sometimes encounter dry conditions. Moreover, it is tempting to suppose that it was possessed by the aquatic ancestors of land Vertebrates. Aquatic animals could afford to possess ureogenesis as an aid to drought survival, provided that it did not result in permanent ureotelism, which would have had negative selection value. When such animals ventured on land journeys, they would already have the enzymes of the Krebs urea pathway,

and the waste nitrogen would be channelled into it in much the same way as in *Xenopus*. Increasingly frequent use of the urea cycle enzymes would then subject them to intensive selection, resulting in an increase in the amounts of these enzymes in the liver.

At a stage comparable to *Xenopus* or *Protopterus*, the early Amphibia would not be able to excrete urea, but only to store it. They would therefore have to return to water periodically to excrete their waste urea. Possibly the acquisition of active urea secretion (Marshall & Crane, 1924; Forster, 1954) was the step which completely freed amphibian excretion from dependence on abundant water supply, and rendered them truly terrestrial.

Lastly, a subsequent loss of the ability to make large quantities of ammonia must be postulated. This would explain why species such as *Rana esculenta* or *Triturus cristatus* do not become ammoniotelic even when living in water.

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CHANGE IN INTRACELLULAR CONCENTRATION OF FREE AMINO ACIDS AS A FACTOR OF EURYHALINITY IN THE CRAYFISH *ASTACUS ASTACUS*

GH. DUCHÂTEAU-BOSSON and M. FLORKIN

Department of Biochemistry, University of Liège, Belgium

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Abstract—1. *Astacus astacus* survives in twice-diluted seawater in spite of the fact that the osmoregulatory mechanism which is active in freshwater maintains the concentration of the blood at an even higher level than that of the diluted seawater.

2. In these conditions, the degree of hydration and the potassium content of the abdominal muscle show only a very slight modification.

3. The adaptation results from an increase of intracellular concentration which is partly due to an increase of free amino acids.

4. While the increase of blood concentration, in a crayfish transferred from fresh water to twice-diluted seawater, amounts to a change in Δ of 0·24°C, the increase in concentration of intracellular amino acids corresponds to 0·15°C, which shows that the free amino acids play an important role in the mechanism of adjustment.

INTRODUCTION

VALUES of the concentrations of fifteen free amino acids (those enumerated in Table 1) in blood plasma and muscle of *Astacus astacus* have been recorded by Camien, Sarlet, Duchâteau & Florkin (1951) and by Duchâteau & Florkin (1954). These determinations, accomplished by microbiological methods, have been realized on dialysates of inactivated homogenized muscles before or after hydrolysis. As the hydrolysis changes the concentration of aspartic and glutamic acids only, the data published by the above authors represent the values for the concentration of free amino acids, except in the case of glutamic and aspartic acids where they correspond to the sum of the free amino acid and of its amide. Similar results have been obtained for another crayfish *Astacus* (*Austropotamobius*) *pallipes* Lereboullet (= *saxatilis* Bell) by Cowey (1961).

In spite of the fact that the crayfish is a typical freshwater form, there are a number of records of crayfish in brackish water (Smith, 1959). In an experimental study by F. Hermann (1931) it was observed that the crayfish *Astacus astacus* can support an increase in concentration of the water up to $\Delta = 1{\cdot}07°C$ where the animals live for weeks, whereas they rapidly die if placed in more concentrated media.

Duchâteau-Bosson, Jeuniaux & Florkin (1961) have pointed out that, in more or less euryhaline marine Invertebrates, the degree of euryhalinity is accounted for either by an adjustment of the intracellular concentration, a regulation in which free amino acids are involved in all cases so far studied, or by a combination of an

osmoregulation of the blood and of an adjustment of the intracellular concentration to the level maintained in the blood as a result of its osmoregulation. Cases in the first category are represented by *Mytilus* (Potts, 1958), *Arenicola marina* (Duchâteau-Bosson, Jeuniaux & Florkin, 1961) and by *Perinereis cultrifera* (Jeuniaux, Duchâteau-Bosson & Florkin, 1961b). These forms have no osmoregulatory mechanism of the blood but, in diluted media, are able to adjust their

TABLE 1—*Astacus astacus* (FED WITH MEAT)
Amino acids, mg/100 ml or 100 g fresh tissue*

	January Plasma	January Muscle	May Muscle		May Muscle		May Muscle	June Muscle	June Muscle	July Muscle
	H	H	NH	H	NH	H	H	H	H	H
1. Alanine	10·2	54·7	91·1	97·5	78·2	91·2	63·7	—	—	60·8
2. Arginine	3·6	945·8	865·4	810·9	739·4	693·7	711·4	831·7	558·2	713·2
3. Aspartic acid	2·3	97·5	16·0	95·0	15·5	77·5	80·5	114·6	90·7	76·6
4. Glutamic acid	29·8	425·0	63·2	519·7	46·8	422·5	462·9	412·7	374·6	301·9
5. Glycine	6·0	253·0	135·4	123·3	124·3	121·1	106·2	139·4	223·5	110·6
6. Histidine	1·1	35·0	65·4	33·0	44·4	45·1	40·7	63·5	23·0	18·9
7. Isoleucine	6·0	17·7	13·5	15·1	10·2	12·3	12·4	17·8	14·6	20·0
8. Leucine	3·0	32·6	23·4	26·0	18·7	19·0	18·9	27·3	24·8	29·4
9. Lysine	3·0	102·0	99·1	92·7	74·6	72·9	88·5	74·9	66·6	84·5
10. Methionine	—	—	20·5	20·9	14·8	15·5	15·5	18·1	13·1	19·6
11. Phenylalanine	1·0	20·9	17·0	17·0	15·1	15·1	14·0	18·1	10·4	9·4
12. Proline	3·3	124·0	187·7	174·8	174·6	168·3	126·1	151·7	168·4	175·5
13. Threonine	3·3	32·5	39·5	38·8	23·9	24·3	22·1	40·3	26·3	25·7
14. Tyrosine	1·7	19·3	23·7	21·5	20·1	16·9	17·4	16·5	10·7	11·7
15. Valine	6·0	25·3	25·7	26·6	23·9	23·6	20·5	27·3	24·8	30·9
Total				2113·0		1819·0	1800·8			1688·7

* Duchâteau & Florkin, 1954.

intracellular concentration to the new concentration of their blood, which is equal to that of the diluted medium (poikilosmotic euryhalines). In the process of adjustment of the intracellular concentration, the amino acids play an important role.

On the other hand, euryhaline forms such as *Carcinus maenas* (Duchâteau & Florkin, 1956; Shaw, 1958), *Eriocheir sinensis* (Duchâteau & Florkin, 1955) and *Nereis diversicolor* (Jeuniaux, Duchâteau-Bosson & Florkin, 1961b) are more euryhaline than those mentioned above. In diluted media, they rely on an osmoregulation of the blood and on an intracellular adjustment to the new blood concentration, realized at least partly by a marked change in the concentration of intracellular amino acids (homeosmotic euryhalines).

When the crayfish lives in freshwater ($\Delta = 0{\cdot}02°C$), the Δ of its blood is maintained at a much higher value (0·8°C) than that of the external medium (Léon Fredericq, 1898). In all the media in which the crayfish survives (i.e. up to a Δ of

about 1·07°C in the external medium) the Δ of the blood is higher than the Δ of the external medium, and the weight of the animal does not change (Hermann, 1931).

In fresh water, the great difference of concentration between the external medium and the cell fluid, which is in osmotic equilibrium with the blood, is compensated by the mechanism of osmoregulation, maintaining the Δ of the

TABLE 2—*Astacus astacus* SERUM OR ABDOMINAL MUSCLES

	Serum mEq/*P*			Muscles mEq/Kg water			Dry weight % freshwater
	Ca	Mg	K	Ca	Mg	K	
Controls in freshwater	21·5	4·3	4·6	7·0	23·6	93·2	18·80
	20·3	3·0	4·8	8·7	20·4	101·0	17·35
	20·7	2·6	4·6	8·8	20·4	95·3	16·96
	22·6	2·8	4·9	7·6	19·3	99·0	16·86
2 × diluted seawater							
15 days	28·8	7·3	3·0	19·3	28·8	99·8	20·64
	27·4	8·0	4·1	17·4	27·1	92·4	19·78
	25·5	6·9	2·9	15·0	22·4	82·6	16·15
1 month	29·5	9·4	6·1	13·7	25·6	109·0	18·90
	30·8	9·6	6·0	14·6	26·4	112·8	19·83
	29·9	12·9	5·4	12·9	23·8	103·1	18·62
3 months	28·0	11·9	6·2	19·3	22·6	93·1	16·28
	29·7	11·1	5·8	13·9	23·1	103·0	17·51

blood at the value of 0·8°C (Δ of the external media = 0·02°C). The intracellular concentration of the crayfish is lower than that of a marine Crustacean such as the lobster, whose cells are in osmotic equilibrium with the blood, which is itself in equilibrium with the seawater. This is partly due, as first pointed out by Camien, Sarlet, Duchâteau & Florkin (1951), to a smaller concentration of free amino acids in the cells.

In a freshwater form such as the crayfish, osmoregulation maintains, in the external media compatible with life, a concentration of the blood higher than that of the external concentration. Osmoregulation therefore becomes a limiting factor of the extension in waters of higher concentration than freshwater, except where the intervention of an increase in intracellular concentration compensates for the increase in the concentration of the blood and avoids extended changes of hydration of the cells.

METHODS

The abdominal muscles have been rapidly removed from several animals, cut into pieces with scissors, pooled and weighed. The tissues (8–16 g) were then placed

in boiling water for five minutes to inactivate the proteolytic enzymes. The suspension has been homogenized in a Waring blender and dialysed for 24 hr in 10 volumes of distilled water, at 0°C, with permanent mechanical agitation. After evaporation, the dialysate has been refluxed at boiling temperatures for 24 hr in 6 N HCl. The amino acids have been determined by microbiological procedures (Duchâteau & Florkin, 1954).

TABLE 3—*Astacus astacus* ABDOMINAL MUSCLE

mM/Kg fresh muscle

Amino acid	Controls in fresh-water, 10–11°C 5/10/55		Twice-diluted seawater, 10–11°C 20/9/55–5/10/55					
1. Alanine	13·3	13·2	24·3	23·1	23·2	12·7	17·4	25·9
2. Arginine	33·9	40·0	53·7	47·5	45·4	38·9	36·9	42·8
3. Aspartic acid	11·4	4·5	9·1	10·7	9·3	8·2	10·1	9·8
4. Glutamic acid	41·8	26·9	33·8	40·6	56·4	56·9	61·2	54·1
5. Glycine	16·2	25·6	24·8	26·3	13·6	22·6	17·7	40·5
6. Histidine	·9	·2	3·1	2·2	1·6	·3	1·8	·8
7. Isoleucine	2·0	1·4	3·1	2·7	3·0	2·6	4·8	4·0
8. Leucine	2·5	1·6	3·4	3·3	4·4	3·7	5·1	3·9
9. Lysine	9·4	1·9	9·8	4·3	10·0	2·6	7·3	8·4
10. Methionine	2·1	1·4	2·5	2·4	1·4	1·4	1·6	1·7
11. Phenylalanine	1·2	·6	·8	·5	1·0	·3	·2	·8
12. Proline	9·0	6·6	21·8	30·3	24·7	16·8	35·1	25·5
13. Threonine	3·7	1·7	4·8	4·3	6·0	5·0	6·8	6·8
14. Tyrosine	1·7	·8	1·3	·8	1·2	·7	·5	1·3
15. Valine	2·5	1·8	3·9	3·8	4·2	3·5	6·2	5·8
Total	151·6	128·2	200·2	202·8	205·4	176·2	212·7	232·1
Mean	139·9		204·0					
Dry weight (mean) % g fresh weight	17·49		18·86					
Total (mM/Kg water)	169·6		252·5					

Amino acid variation 252·5 mM − 169·6 mM = 82·9 mM
" " " due to dehydration = 252·5 mM − 248·3 mM = 4·2 mM
" " " = 82·9 mM − 4·2 mM = 78·7 mM = 0·15°C

RESULTS AND DISCUSSION

Table 2 shows the changes in Ca, Mg and K content in twice-diluted seawater, measured in the blood and in abdominal muscles, after a more or less prolonged stay in the concentrated medium (15 days–3 months). The degree of hydration and the potassium content of the muscles show only a very slight modification after two weeks (Tables 2 and 3), in spite of the fact that the Δ of the water has been changed from 0·02°C to 0·95°C, and the Δ of the internal medium (Hermann, 1931) from 0·8°C to 1·04°C.

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As shown in Table 3 there is an increase in concentration of the total of the fifteen amino acids. This mean increase amounts to 82·9 mmoles of amino acids/Kg of water (252·5–169·6). Of this, the slight amount of dehydration accounts for 4·2 mmoles only. The change in concentration of amino acids amounts to 78·7 mmoles/Kg of water, which corresponds to a change of Δ of 0·15°C. As the increase of the blood concentration, in these circumstances, corresponds to 0·24°C (1·04°–0·8°), the increase in the concentration of the 15 amino acids determined in the present study compensates osmotically for more than half the increase of blood salts concentration. The variations of concentration of the inorganic constituents of the muscle fibres account for only an insignificant contribution to the compensatory mechanism (for instance, 0·017°C in the case of Ca). Among the amino acids, total glutamic acid (glutamic acid + glutamine), proline and alanine appear to be responsible for the main part of the increase of intracellular osmotic pressure in the concentrated medium.

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THE INFLUENCE OF THE SALINITY OF THE MEDIUM ON RESPIRATION IN ISOLATED GILLS OF THE CLAM *DREISSENSIA POLYMORPHA* (PALL.)*

J. BIELAWSKI

Department of Animal Physiology, Poznań University, Poland

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Abstract—1. The respiration of the isolated gills of *Dreissensia polymorpha* (Pall.) was investigated by a polarographic method in flowing media of different chemical composition.

2. In tap water the gills respire with an unchanged intensity for at least 22 hr.

3. The change in salinity from tap water to 3·6‰ causes an increase of respiration for about 4 hr and then the respiration rate gradually returns to its initial value. With 7·2‰ salinity the increase of O_2 consumption is slight. An increase of salinity of 14·4‰ causes a decrease in the rate of respiration.

4. Replacing tap water with 3·6‰ NaCl solution causes a temporary increase of respiration followed by a rapid decrease to a low value. KCl and $MgCl_2$ addition to NaCl solution slow down this fall in respiration rate. The presence of $CaCl_2$ with NaCl has no influence on the short period of increased respiration, but it delays the fall in respiration rate for about 7 hr.

5. None of the cation combinations brought about normal respiration of the isolated gills.

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INTRODUCTION

INVESTIGATIONS on the influence of salinity on aquatic animals have shown that in many invertebrates a change of salinity causes a change in the intensity of respiration. Salinity may influence the organisms in two ways, namely by osmotic concentration and by chemical components dissolved in water.

In the majority of animals the relation between salinity and respiration is a complex phenomenon, because the respiring cells are not situated in the surrounding water, but in the body fluids which more or less differ from it.

The preservation of an independent internal medium requires some work, and the energy for it is supplied by the processes of respiratory metabolism. As follows from the investigations cited in an earlier work (Bielawski, 1960) the amount of energy necessary for preserving the independent internal medium, following a change of water salinity does not cause any perceptible change in the intensity of respiration.

Some euryhaline animals, such as *Carcinus maenas* (Schlieper, 1929) or *Gunda ulvae* (Beadle, 1931), increase the intensity of respiration as the osmotic concentration of water decreases. There are, however, a number of animals in which

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respiration does not depend on salinity. Not only homoiosmotic animals, in which the osmotic concentration of body fluids varies slightly, such as *Eriocheir sinensis* (Schwabe, 1933) or *Artemia salina* (Gilchrist, 1956; Croghan, 1958a), but also the animals in which the internal medium varies considerably under the influence of the changes in salinity of surrounding water, e.g. *Theodoxus fluviatilis* (Lumbye, 1958; Bielawski, 1960; Neumann, 1960), belong to this group.

Chemical composition of the medium is another factor influencing the intensity of respiration. Thus in the snail *Viviparus fasciatus* transferred from fresh to diluted isotonic artificial sea water one can observe an increase of oxygen consumption (Obuchowicz, 1958). Karpevich's (1958) investigations are an even better illustration of the dependence of respiration on ion content in the medium. The animals of the same species respire with different intensity in brackish waters of the same salinity, but coming from different natural sources. Also a change in salinity in some instances involves a change in respiration, whilst in others it does not, depending on chemical composition of the water.

Isolated organs or tissues can change their intensity of respiration depending on the ionic content of the medium. Isolated gills of *Mytilus edulis* (Schlieper, 1936), *Carcinus maenas* (Pieh, 1936) and *Venus mercenaria* (Hopkins, 1949) respire with a greater intensity in NaCl solution than in sea water isotonic with it. The respiration of *Venus mercenaria* gills does not increase if the solution contains both NaCl and $CaCl_2$. Hopkins explained this influence of the calcium as an antagonistic action of Na and bivalent ions, but according to Schlieper (1936) the gills of *Mytilus edulis* respire with increased intensity in the solution of $CaCl_2$ alone.

Experiments were carried out on the isolated gills of the clam *Dreissensia polymorpha* in order to get some closer knowledge of the mechanism of the influence (a) of water salinity and (b) of separate ions on respiration. These particular gills are especially well suited to the investigations of that kind, as they respire constantly for 24 hr in the medium characterized by very low content of ions, such as tap water (Wernstedt, 1944). The intact animals do not change their intensity of respiration even if the salinity of water varies considerably (Karpevich, 1947), but respiration does depend on the chemical composition of the medium (Karpevich, 1958).

MATERIAL AND METHODS

The clams were collected in Kierskie Lake near Poznań, in the vicinity of the bank. They were brought to the laboratory and placed in glass basins filled with aerated tap water at room temperature (*ca.* 20°C). The water was changed approximately every 5 days in order to prevent excessive growth of the bacteria. Animals used for experiments had been kept in these conditions for 2–18 days.

As the intensity of respiration in the clams in question depends on their size (Wesemeier, 1960), only the specimens with the shells 27 to 29 and (exceptionally) 26 mm long were used for experiments. Also all specimens whose gills were attacked by any noticeable parasites were rejected.

The metabolism of clam gills varies somewhat depending on the season of the year (Hopkins, 1949). For that reason the approximate date of collecting the clams was given with the description of the experiments.

Preliminary investigations were made on clams kept in winter in basins with a continuous flow of tap water. These investigations have shown that addition of glucose increases the intensity of respiration of isolated gills. For that reason immediately after excision the gills were kept in solutions containing glucose in the concentration of 10^{-2} M/l.

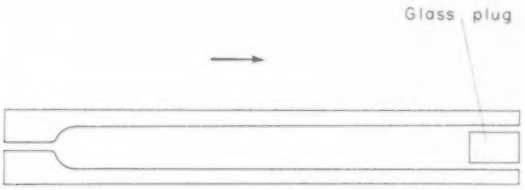

FIG. 1. A diagrammatic cross-section of the respiratory vessel. The arrow indicates the direction of the water flow.

The measurements of respiration were made using the method described in Bielawski (1961) and the respiratory vessel has been adapted to the investigations of isolated gills. The diagram of the vessel is shown in Fig. 1. It is made of a glass tube passing in one end into the capillary, and equipped at the other end with a glass stopper or a small ball of silver wire, which keeps the gills inside the vessel but allows water to flow. The inside diameter of the vessel was 3·5 mm, and the volume 0·25 ml. The small volume and the elongated shape of the vessel assure a good exchange of gases between the respiring gills and flowing water.

The water flowing through the vessel amounted to 3 ml/hr, and thus a quantity of water equal to the volume of the vessel flowed through it in 5 min. The flow rate was kept constant.

After isolation from the organism the gills were placed in a vessel filled with tap water and thus slime was removed. Four gills from one or two specimens were subsequently placed in the respiratory vessel, through which tap water was flowing. The gills respired in that water for 2 hr, and then the medium was changed according to the requirements of the given experiment.

In order to avoid calculating the results per size of gills, the intensity of respiration was presented as per cent of the average value obtained during the first two hours in tap water. In this way also the error resulting from differences of intensity of respiration between individual specimens was diminished. The fact that one need not calibrate the respirometer is another advantage of such presentation of results. It is sufficient to know that the diffusion current is a linear function of the dissolved oxygen content.

The curves in Figs. 4, 5 and 6 represent the mean values of three experiments. The oxygen consumption was read from polarograms, starting from the moment when the medium was changed. The readings were taken every 0·25 cm in the

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beginning, then every 0·5 cm, every 1 cm and at last every 5 cm. The frequency of the readings was higher at the beginning, as rapid changes in respiration usually follow immediately the change of medium. The light-sensitive paper on which the deflections of the galvanometer were registered moved at 2·28 cm/hr. The comparable readings of three analogous polarograms were summed and the mean values calculated, and the curves shown in the figures are lines passing through these mean values.

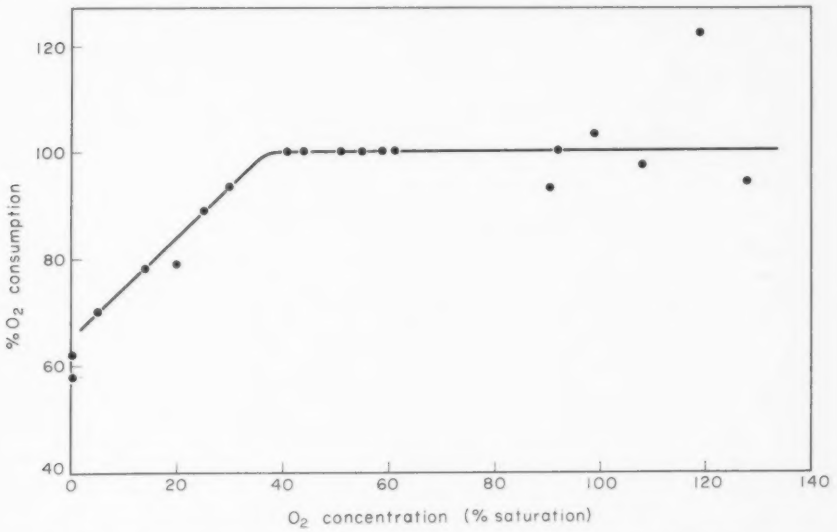

FIG. 2. The influence of the oxygen content in water on the intensity of respiration in isolated gills. The intensity of respiration is presented in per cent of O_2 consumption in aerated tap water (100 per cent saturation). The oxygen content in the water flowing out of the respiratory vessel is presented on the abscissa.

Every medium in which the gills were placed was aerated for at least 15 min at $20 \pm 0{\cdot}1°C$. All the experiments were made at the same temperature.

Artificial sea water of different salinity was prepared as described in the earlier paper (Bielawski, 1960). Analytical grade reagents, dissolved in glass-distilled water, were used for the investigations on the influence of separate ions on respiration.

pH measurements were made using a glass electrode.

RESULTS

(1) *The influence of oxygen concentration*

The oxygen concentration of the environment may be a factor influencing the intensity of respiration (Bielawski, 1960). In order to eliminate this factor a number of measurements were made to determine the critical oxygen concentration.

The isolated gills were placed in a respiratory vessel into which air-saturated tap water was flowing. The oxygen concentration of this water was termed here as 100 per cent. The oxygen concentration was varied twice every 2 hr, the highest concentration being 170 per cent.

Water with the known oxygen concentration was prepared in the same way as for calibration of the respirometer (Bielawski, 1961).

The results of determination are shown in Fig. 2.

The results show that the critical O_2 concentration in the water flowing out of the vessel is 40 per cent. When the oxygen concentration is lowered below this value the respiration rate is slowly diminished.

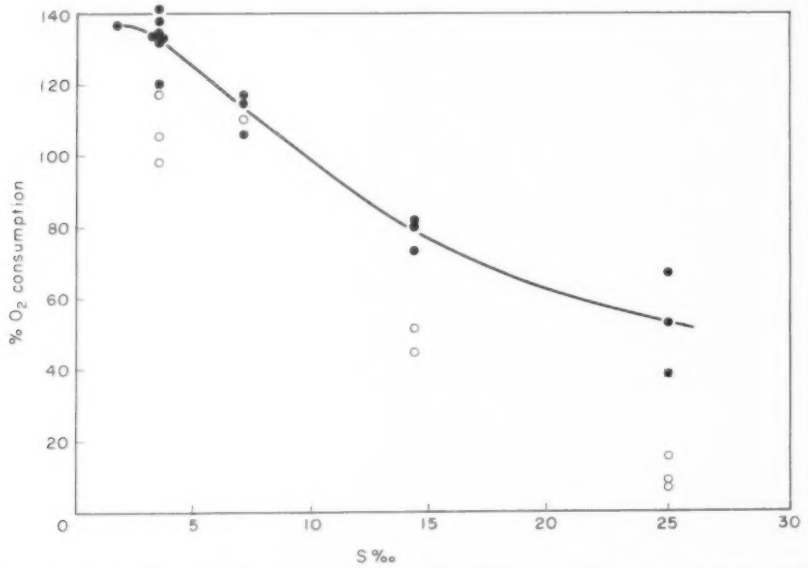

FIG. 3. The influence of the salinity on the respiration intensity in isolated gills. The intensity of respiration is presented as per cent of that shown by the gills in tap water. ● = Intensity of respiration during the first two hours after the change of salinity, ○ = intensity of respiration after 18 hr in the given water.

(2) *The influence of salinity*

The clams for these experiments were collected in May and June.

After 2 hours' respiration by the gills in fresh water, the water was replaced by water of a known salinity and the respiration rate was measured for 20 hr.

The results are shown in Figs. 3 and 4. Each point (●) in Fig. 3 represents the mean respiratory rate of the gills over the first two hours in the same medium. The period of time following immediately a change in salinity has been omitted because respiration is disturbed by a sudden change of the medium. The circles (○) in Fig. 3 show the rate of respiration after 18 hr in the given water.

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One can infer from the curve presented in Fig. 3 that a salinity of 10‰ does not affect the intensity of respiration. A lower salinity causes a temporary increase of the oxygen consumption, and a higher one—its decrease.

Fig. 4 shows the mean curves respresenting the dependence of the respiration intensity on the time during which the gills have remained in the given medium. The zero time in the diagram shows the moment at which one started to supply water of the final salinity.

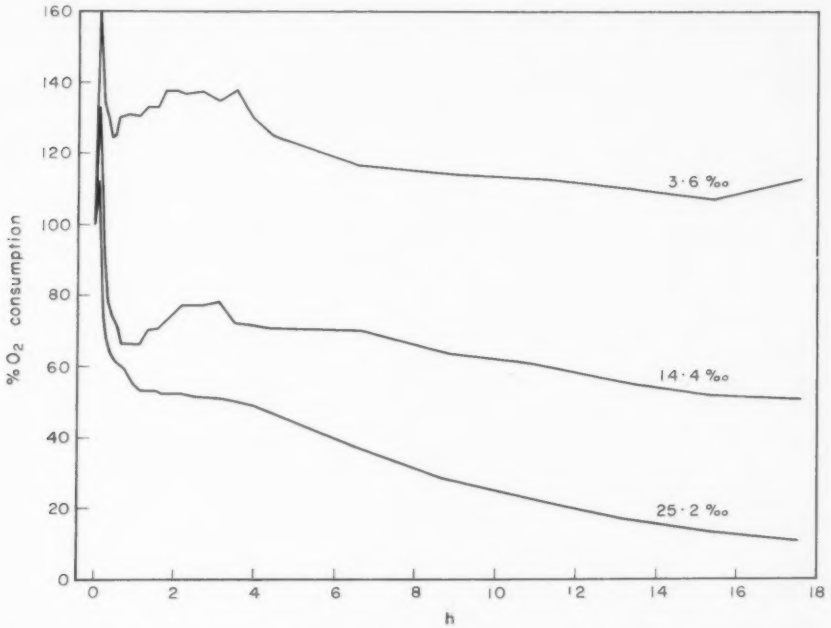

FIG. 4. The dependence of the respiration intensity of isolated gills on the time of keeping in the water of a given salinity. The intensity of respiration is presented as in Fig. 3. Zero time = the moment of application of the final salinity. The curve representing the intensity of respiration at 14·4‰ salinity is the mean of two measurements.

It has been already mentioned that the intensity of respiration increases for a short time immediately after each change in salinity of the environment, and then it falls to a certain level. This increase cannot be explained by the short pause in water supply while water is changed.

In water of 3·6‰ salinity the respiration is equal to 130 per cent of the initial value during the first four hours and then it gradually drops to about 100 per cent.

With 7·2‰ salinity the respiration slightly exceeds 100 per cent during the first two hours, and presumably retains this level for 18 hr.

A further increase of salinity (14·4‰) causes decrease in oxygen intake to about 75 per cent. The gills respire with about the same intensity for *ca.* 7 hr, and then one can observe a slow decrease in oxygen consumption.

In 25·2‰ salinity one can observe a destructive influence on respiratory mechanism after four hours, as after that time the respiration falls rapidly to a low value. On the other hand, during the first four hours the respiration is constant and amounts to 50 per cent of that in tap water.

(3) *The influence of different cations*

In order to determine the influence of different cations, and also the ratio of univalent to bivalent cations, a number of measurements of the gill respirations were made in solutions with different cation content. The final concentration of

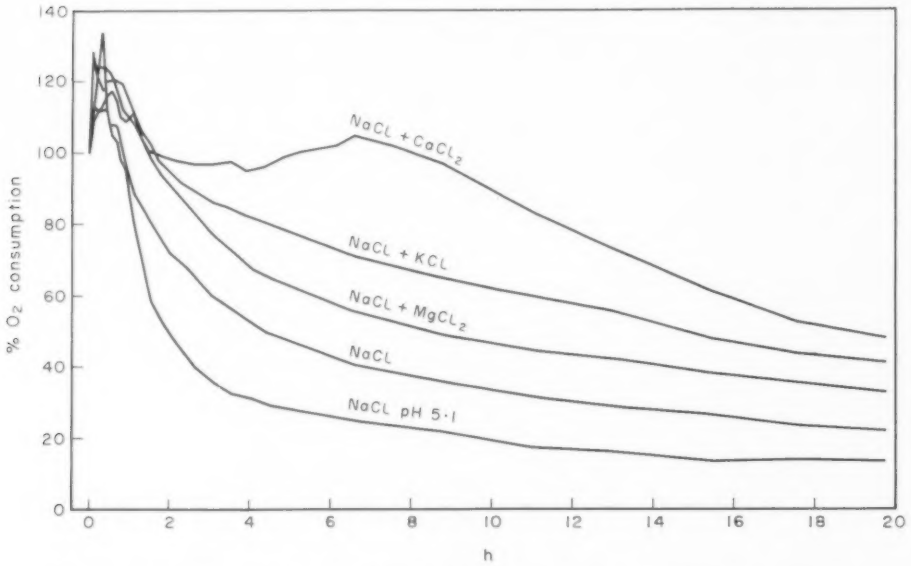

FIG. 5. The influence of the time of keeping of the isolated gills in the solutions of different salts on the intensity of respiration. The final concentration = 3·6‰. The intensity of respiration is presented as in Fig. 3. The curve representing respiration in NaCl solution of pH 5·1 is the mean of two measurements.

solutions in these experiments was always 3·6‰. When the gills are left in artificial sea water of this concentration for a long time, they respire with about the same intensity as in tap water (Fig. 4).

NaCl is the main salt component of sea water, and so this salt was used in all cases, together with the addition of other cations. The concentration of separate cations was such as to correspond to their ratio to Na in artificial sea water.

The clams for experiments were collected in July.

The solutions of salts were adjusted to pH $7{\cdot}9 \pm 0{\cdot}1$ with Na_2CO_3. The pH of tap water in which the gills respired during the first two hours was 7·9. The experimental NaCl solution showed pH 5·1 by the presence of H_2CO_3.

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The intensity of respiration increases immediately after replacing tap water with any of the experimental solutions. The gills respire with increased intensity for a comparatively short time, and then the rate drops (Fig. 5). During that time the variations in oxygen consumption are so high that the differences between separate curves are accidental. The essential difference appears only between the curves showing respiration in NaCl and other solutions. In the former the respiration drops much faster.

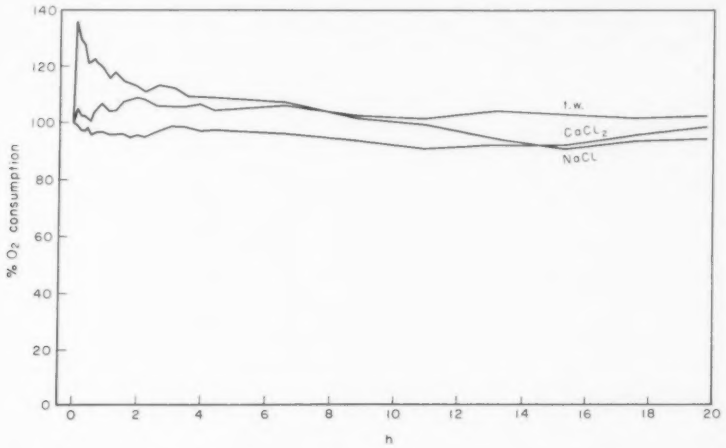

FIG. 6. The influence of the increase of Na or Ca ions concentration in tap water on the intensity of respiration. The intensity of respiration is presented as in Fig. 3. t.w. = O_2 consumption in tap water.

Respiration in NaCl solution drops quickly after a temporary increase so that in about 1 hr it returns to the initial value; reaches 50 per cent after 4 hr and 20 per cent after 20 hr. The addition of separate cations to the NaCl solution diminishes the fall in respiration rate.

All the curves in Fig. 5 are similar, with the exception of those representing respiration in a solution containing Ca ions. In this case after an initial increase lasting about 2 hr the respiration remained at its initial value for a further 7 hr, and only then was there a decrease. After 20 hours' measurements the respiration was still comparatively high and reached 50 per cent of the initial value.

As can be seen from the quoted data none of the ion combinations tried here was sufficient for assuring normal functioning of respiratory mechanism.

Experiments on the oxygen consumption of isolated gills in tap water and in water with increased concentration of NaCl or $CaCl_2$ were made in order to determine if the ratio of uni- and bivalent cations had any essential influence on respiration.

The solutions were prepared as follows: a 3·6 per cent solution of the given salt was diluted with tap water to the concentration of that salt in artificial sea water of 3·6‰.

The animals for these experiments were collected in September and October.

The results are presented in Fig. 6. They show that the addition of the NaCl solution caused an immediate increase of respiration, which after some time returned to the initial value. (It is somewhat different from the artificial sea water of the same concentration.) $CaCl_2$, on the other hand, does not cause any perceptible changes. After about 3 hr no essential differences between the three curves can be seen. Hence one may conclude that the univalent and bivalent ion concentration has no long-term influence on the respiration of isolated gills of the clams.

The results marked in Fig. 6 also confirms Wernstedt's (1944) conclusions that gills respire in tap water at the same rate for 24 hr.

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DISCUSSION

Many experiments have shown that there is an interchange between the animal and its environment (Lilly, 1955; Jørgensen & Dales, 1957; Croghan, 1958b). The ion concentration in the organism and the environment is the result of a certain steady state. Thus transferring the worm *Gunda ulvae* (Pantin, 1931) or *Nereis diversicolor* (Jørgensen & Dales, 1957) from normal to diluted sea water causes diffusion of ions from the organism into the environment till a new steady state is reached.

It follows from the above that the investigations of changes of osmotic concentration and of ionic content of water should be made in a flowing medium. In such conditions both ions and products of metabolism eliminated by the animal will be removed.

Isolated tissues or organs also show some osmoregulatory properties (Pieh, 1936; Koch, Evans & Schicks, 1954), and their metabolism often depends on osmotic concentration and ionic composition of the medium (Pieh, 1936; Schlieper, 1936; Peters, 1936; Hopkins, 1949).

The comparison of the results of the present experiments with analogous investigations of other authors is rather difficult, as all the earlier measurements of respiration of isolated tissues were made in the vessels without any flow of the medium.

The isolated gills react to a change of salinity like the whole clams investigated by Karpevich (1957), i.e. salinity up to a certain value does not influence the intensity of respiration. Fig. 3 shows that salinity higher than 10‰ causes the decrease of the intensity of gill respiration. 10‰ is also the maximum salinity of water from the Caspian Sea, in which *D. polymorpha* can live for a longer time.

A similar relationship to that of *D. polymorpha* between respiration and salinity is known in many animals, e.g. *Mya arenaria* (Schlieper, 1929), *Eriocheir sinensis* (Schwabe, 1933), or *Theodoxus fluviatilis* (Lumbye, 1958; Bielawski, 1960),

and can be considered as one of the symptoms of accommodation to life in conditions of different salinity.

The different intensity of respiration of animals in brackish waters from different natural sources is explained by Karpevich (1958) by different ratios of uni- and bivalent cations. But it can be inferred from the present investigations that the cation ratio has no lasting influence on the respiration of isolated gills, because an increase of concentration of Na ions in tap water changes respiration for only a short time, and the increase of Ca ions concentration does not cause any perceptible changes.

The absence of antagonistic influence of uni- and bivalent cations on respiration is also proved by the fact that K ions modify the influence of NaCl more than do Mg ions (Fig. 5).

The results of investigations on the influence of different salts on the respiration of isolated gills described in the present work differ from similar investigations of other authors. Isolated gills of invertebrates transferred from the water of a given salinity to a NaCl solution isotonic with it have an increased oxygen consumption (Schlieper, 1936; Pieh, 1936; Hopkins, 1949). But no increase of respiration intensity was observed in the gills of *Venus mercenaria* when the solution contained both NaCl and $CaCl_2$ (Hopkins, 1949).

The gills of *D. polymorpha* respire with an increased intensity in NaCl solution for one hour, and then the intake of O_2 drops rapidly. The presence of K, and (in a lesser degree) Mg ions diminishes the rate of this decrease. The addition of Ca, on the other hand, has a marked effect. The presence of this ion in the NaCl solution considerably retards the decrease in respiration rate.

The investigations on osmotic regulation have shown that Ca diminishes the permeability of the animal body surface (Pantin, 1931; Beadle, 1934; Ellis, 1937). These facts suggest that Ca influences respiration by diminishing the permeability of cell membranes.

Ca could diminish the permeability of cell membranes and thus decrease the speed of changes occurring in the cells.

A more detailed knowledge of the mechanism of the gill respiration is required to confirm the above view concerning the influence of Ca on gill respiration and to elucidate the role of the remaining cations.

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THE USE OF A SOLID PLATINUM ELECTRODE FOR CONTINUOUS RECORDING OF THE RATE OF RESPIRATION*

J. BIELAWSKI

Department of Animal Physiology, Poznań University, Poland

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Abstract—1. A polarographic method of measuring respiration rates of tissues is described.

2. The electrodes are platinum cathode and silver–silver oxide anode; both being in 0·5 N KOH. The electrode surface is enclosed by a 0·04 mm polythene membrane.

3. The electrodes function satisfactorily in a flowing current of water, e.g. 3 ml/hr.

INTRODUCTION

IN INVESTIGATIONS on the respiration of aquatic animals the polarographic method is now being applied more and more frequently. One of its advantages is that it allows one to record continuous measurements (Spoor, 1948; Wood, 1953; Briggs, Dyke & Knowles, 1958).

This method is suitable when water flows through a vessel containing the examined animal. The rate of respiration is calculated from the difference between the oxygen concentration in water flowing into and out of the respiratory vessel.

The dropping mercury electrode, which has been used in previous experiments (Bielawski, 1959), has the disadvantage that the size of the drop can be changed only within small limits.

During measurements oxygen is removed from the medium by the cathode. The amount of the removed oxygen is proportional to the intensity of the diffusion current. In investigations on very small animals where the flow of water must be very slow and measurements are recorded continuously, this loss of oxygen may cause a relatively great error. For example, if air-saturated water flows at a speed of 3 ml/hr, the loss of oxygen at the dropping cathode amounts to about 15 per cent.

The intensity of the diffusion current and hence the escape of oxygen from the examined water may be decreased by using a solid cathode having a small surface.

Until recently it was not possible to use a solid cathode for prolonged measurements of respiration because natural waters contain substances which precipitate on the cathode and change the intensity of the diffusion current. One can avoid this by separating both electrodes from the examined water by means of a membrane that is permeable to oxygen but is impermeable to water and ions (Connelly, 1957;

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Krog & Johansen, 1959; Kreuzer, Harris & Nessler, 1960). Such a device is convenient since it allows examination of solutions containing no electrolytes because the electric current does not flow directly through the examined solution.

The most frequently used solid cathode is a platinum wire sealed in glass, but it is not easy to make such an electrode since even minute cracks in the glass cause faulty results. On the other hand, good results are obtained by a platinum plate or wire sealed in epoxy casting plastic (Carritt & Kanwisher, 1959).

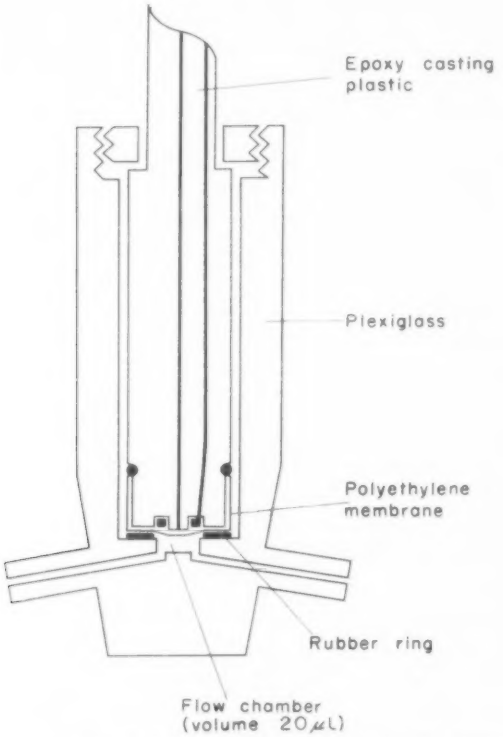

FIG. 1. A diagram of the flow chamber with electrode assembly.

A convenient reference electrode is metallic silver covered with silver chloride. When used for a long time such an electrode becomes brown covered with silver oxide. This process is accompanied by a change in the potential of the electrode. Hence Carritt & Kanwisher (1959) used metallic silver covered with silver oxide as the reference electrode, and KOH as electrolyte.

By means of polarographic methods and using a solid electrode it is possible to make prolonged, continuously recorded measurements without any essential changes of the properties of the electrodes.

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DESCRIPTION OF THE APPARATUS

The construction of the apparatus is in general similar to that described in the previous paper (Bielawski, 1959). The water from the Mariotte flask flows through the respiratory vessel containing the examined animal or tissue and further through the polarographic chamber into the device for examining the speed of the flow.

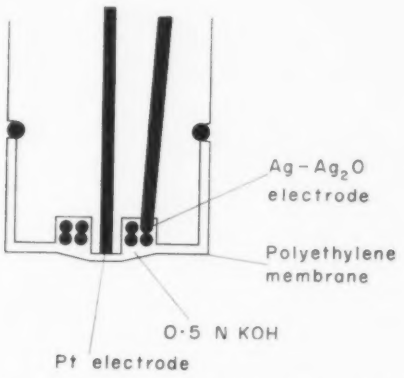

FIG. 2. A diagram of the electrode assembly.

The essential difference is the construction of the polarographic chamber which is illustrated schematically in Figs. 1 and 2. It resembles the chamber used by Reeves, Rennie & Pappenheimer (1957) but is strictly adapted to the measurement of slowly flowing water; of the order of 3 ml/hr.

The cathode is a platinum wire 0·75 mm in diameter sealed in epoxy casting plastic. The surface of the electrode is polished. Around the cathode a groove is bored to hold a silver wire covered with Ag_2O. This wire, the anode, is joined with the positive pole of the source of the current. Both electrodes are enclosed with 0·5 N KOH in a thin polyethylene membrane 0·04 mm thick.

In order to assure the speedy recording of respiratory changes by the electrodes, the water from the respiratory vessel should pass as quickly as possible into the polarographic chamber, and therefore all the tubes should have the smallest possible lumen. The volume of the polarographic chamber should also be very small (about 20 μl). This ensures a steady state of current to become quickly established after a change in the oxygen concentration of the inflowing water. Such a steady state is soon stabilized if only a small amount of electrolyte surrounds the electrodes.

The speed of oxygen diffusion through the membrane separating the cathode from the flowing water depends to a considerable extent upon temperature (Krog & Johansen, 1959; Carritt & Kanwisher, 1959) and so the polarographic cell is placed in a water bath with temperature fluctuations not exceeding $\pm 0{\cdot}1°C$.

During measurements of respiration the water from the Mariotte flask flows parallel into two respiratory vessels and then into the polarographic chambers. Only one of the first mentioned contains the examined animal or tissue, the other being the control vessel.

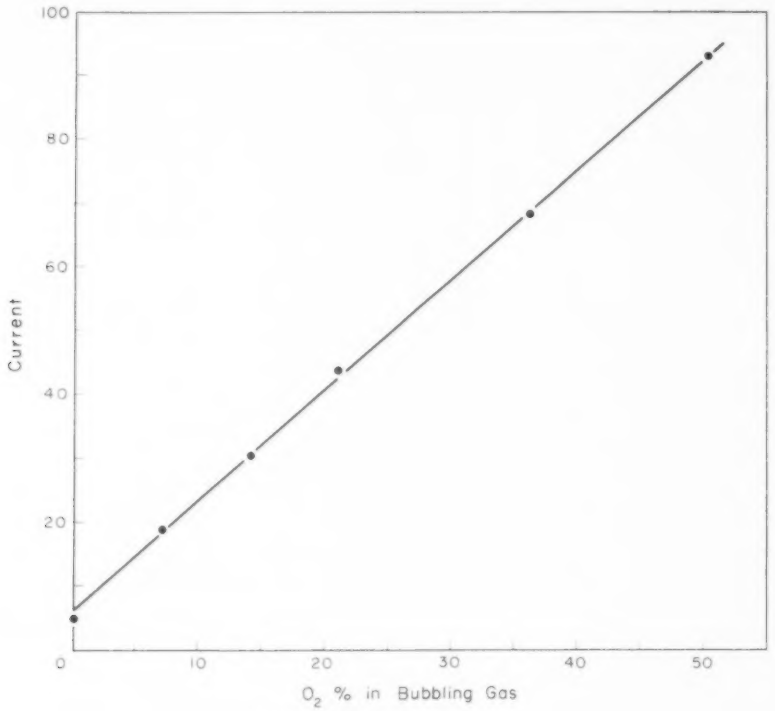

FIG. 3. Calibration curve.

Directly after applying current to the electrodes the intensity of the current diminishes for about 20 minutes and then it remains constant (Carritt & Kanwisher, 1959). This phenomenon makes it impossible to switch the current for a short time to the circuit of the control chamber. For this reason tension is constantly applied to the electrodes in both chambers whereas the galvanometer is switched successively to the circuit of the measuring and control chamber.

$-0{\cdot}7$ V was accepted as the most suitable potential against the $Ag–Ag_2O$ electrode.

CALIBRATION

Calibration consists in determining the relationship between the dissolved oxygen concentration and the diffusion current. Water with a known oxygen concentration was prepared in the following way: A known volume of commercial nitrogen of known temperature and tension was mixed with a corresponding

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volume of air or commercial oxygen. This mixture of gases was subsequently passed through water flowing into the polarographic chamber.

Fig. 3 illustrates the calibration curve which is a straight line not passing through zero. When water saturated with nitrogen passes through the polarographic chamber, the galvanometer records a certain current. This current is caused by the presence of a small amount of oxygen in commercial nitrogen, on the one hand, and, on the other, by a diffusion of oxygen through rubber connecting the glass tubes. As the flow of the water was of the order of 3 ml/hr, the influence of O_2 diffusion through the rubber joinings could not be eliminated. Hence it is important that calibration should be made in conditions similar to those in measurements.

Investigations with the use of enclosed electrodes have shown that air-saturated solutions at the same temperature yield an identical intensity of the diffusion current. This is the case even if the oxygen concentration in these solutions varies considerably, e.g. Ringer's solution and blood (Reeves, Rennie & Pappenheimer, 1957; Krog & Johansen, 1959; Kreuzer, Harris & Nessler, 1960). It is also confirmed by the measurements in artificial sea water at different salinity.

This phenomenon is due to the fact that the speed of oxygen diffusion through the polyethylene membrane does not directly depend on the difference of oxygen concentration in the solutions on both sides of the membrane. It depends, on the other hand, upon the difference in the partial pressure of oxygen of these solutions. We thus measure directly the percentage of oxygen saturation in the solution.

For measuring O_2 concentration in solutions of different salinity, it suffices then to determine the calibration curve for a solution with salinity equal to O and to multiply the values obtained for water with salinity equal to S by the quotient

$$\frac{\alpha S}{\alpha O}$$

αO = Bunsen absorption coefficient of O_2 in water with salinity equal to O
αS = Bunsen absorption coefficient of O_2 in water with salinity equal to S

The results thus obtained have an error since the intensity of the diffusion current is influenced by other factors acting on the speed of oxygen diffusion, e.g. viscosity. In the case of sea water the error is so small that it may well be neglected.

The flow rate of water near the electrodes increases the speed with which oxygen penetrates the surface of the polythene membrane and thus increases the intensity of the diffusion current. If the intensity of the current is sufficiently low and the loss of oxygen in water relatively small, the influence of the flow rate is insignificant. A change of flow rate from 3 ml/hr to 9 ml/hr produces an increase in the intensity of the current by 3·2 per cent.

Continuous measurements lasting 24 hr have shown that during this time the fluctuations in the intensity of the current were slight. In addition, the oscillations in the intensity of the current were identical for both polarographic chambers. Hence it results that where the oxygen concentration in the flowing water is constant, the diffusion current is not subject to changes either.

This method and type of electrode has been applied to measurements of the rate of respiration of the isolated gills of the clam, *Dreissensia polymorpha* (Bielawski, 1961).

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THE SUCCINIC DEHYDROGENASE LEVELS OF THE PECTORAL MUSCLES OF A FEW REPRESENTATIVE TYPES OF BIRDS AND A BAT IN RELATION TO THE FIBRE DIAMETER, MUSCLE WEIGHT AND BODY WEIGHT

J. C. GEORGE and C. L. TALESARA

Division of Animal Physiology and Histochemistry, Department of Zoology, M.S. University of Baroda, Baroda, India

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Abstract—1. Succinic dehydrogenase was estimated quantitatively in the pectoral muscles of a few representative types of birds exhibiting various modes of flight and a bat for comparison.

2. In all the birds and the bat the pectoralis major muscle showed higher activity than the pectoralis minor which shows that of the two, the former muscle is metabolically much more active and highly evolved.

3. The pectoralis major muscle of the rosy pastor, sparrow, bat, parakeet and pigeon showed higher concentrations of succinic dehydrogenase as compared to the other birds studied whereas the fowl, a non-flier, showed the least enzyme concentration.

4. In the birds studied a reciprocal relationship was found between the enzyme activity of the pectoralis major muscle and the body weight, fibre diameter and weight of the pectoralis major muscle.

INTRODUCTION

MUSCULAR tissue in general exhibits a wide range of morphological and biochemical variations. That the homologous muscles of animals belonging to the same group may show great variations in microscopical structure as well as function, has been shown in the flight muscles of insects (Tiegs, 1955; George & Bhakthan, 1960a and 1960b) and in the breast muscles of birds (George & Jyoti, 1955; George & Naik, 1957, 1959a and 1959b). While studying histochemically the distribution pattern of certain oxidizing enzymes in the pectoralis major muscle of the rosypastor (*Pastor roseus*), goose (*Anser albifrons*) and fowl (*Gallus domesticus*), it was suggested that a correlation between structure and function could be derived in terms of the body weight, muscle weight, fibre architecture, fibre diameter, metabolite load and certain cell inclusions against the nature and mode of flight (George & Talesara, 1961a). In order to put this suggestion to further test it was thought desirable to study the concentration of SDH (succinic dehydrogenase) in the pectoral muscles of a few representative types of birds exhibiting different modes of flight and a bat for comparison. SDH was chosen for the present investigation since this enzyme is one of the prime movers of oxidation in the metabolic mill. It may be mentioned here that though considerable amounts of data have accumulated in recent years on the respiratory enzyme levels in the homologous tissues of mammals of

different sizes (Adolf, 1949; Krebs, 1950; Bertalanffy & Prozynski, 1951; Kunkel & Campbell, 1952; Fried & Tipton, 1953; and Kunkel *et al.*, 1956), comparatively little is known about this aspect in the case of birds and hence the justification for the present investigation.

MATERIALS AND METHOD

The SDH activities in the pectoralis major and minor muscles of the following birds and a bat were quantitatively assessed:

Rosy Pastor (*Pastor roseus*)
House Sparrow (*Passer domesticus*)
Green Parakeet (*Psittacula krameri*)
Pigeon (*Columba livia*)
Common Myna (*Aeridotheres tristis*)
Cattle Egret (*Bubulcus ibis*)
Common Babbler (*Argya quadrata*)
Crow Pheasant (*Centropus sinensis*)
Fowl (*Gallus domesticus*)
Bat (*Hipposideros speoris*)

All the animals studied except the fowl and the pigeon were either trapped or shot with an air rifle. The weight of each of the animals was recorded prior to decapitation. The pectoralis major muscle of one side was removed completely and weighed. Since the ratio of the distribution of the different types of fibres in a mixed muscle varies from region to region (George & Talesara, 1960, 1961b; Talesara, 1961), for the estimation of SDH activity, a strip of muscle (with its entire depth) cut along the direction of the fibres was removed quickly from the middle of the pectoralis in each case. The muscle strips removed from the pectoralis major and minor muscles were homogenized separately in cold distilled water in mortars chilled previously. 1 ml of approximately 5 per cent homogenate prepared in each case was used for the estimation of SDH activity. The enzyme activity was estimated according to the procedure of Kun & Abood (1949) using TTC (triphenyl tetrazolium chloride) as the hydrogen acceptor. The incubation mixture in each case contained 0·5 ml of 0·1 M phosphate buffer of pH 7·4, 0·5 ml of 0·5 M sodium succinate, 1·0 ml of the muscle homogenate and 1·0 ml of a freshly prepared 0·1 per cent solution of TTC in a total volume of 3 ml. The enzyme activity is expressed in μg of formazan formed/mg dry weight of the muscle/30 min at 37°C under aerobic conditions. The dry weight of the homogenate used was determined on 1·0 ml duplicate samples dried to constant weight at approximately 100°C.

Simultaneously a small piece of muscle was cut from the pectoralis major muscle and used for determining the diameter of the fibres. Fresh frozen sections of this muscle piece were mounted in 50 per cent glycerol solution on microslides and the diameter of the fibres for each preparation was recorded using an ocular scale and a micrometer slide.

RESULTS

The body weight, the weight and fibre diameter of the pectoralis major muscle and the SDH activity of the pectoralis major and minor muscles of the different birds and a bat studied are given in Table 1. In each individual bird and the bat, the pectoralis major muscle showed considerably higher concentrations of SDH than that of the pectoralis minor muscle except in the case of the fowl, where the difference in the enzyme activity was not much marked. The mean diameter of the fibres in the pectoralis major muscles was found to vary with the size of the muscle.

TABLE 1—SHOWING THE SUCCINIC DEHYDROGENASE ACTIVITY IN THE PECTORALIS MAJOR AND MINOR MUSCLES OF DIFFERENT BIRDS AND A BAT

Animal	No. of animals used	SDH activity in μg formazan/mg dry muscle/30 min		Body weight in g	Pectoralis major muscle†	
		Pectoralis major	Pectoralis minor		Fibre‡ diameter in μ	Weight in g
Rosy Pastor	20	11·50 ± 2·436	4·90 ± 0·555	70	32·12	7·5
House Sparrow	6	10·04 ± 0·871	4·06 ± 0·588	20	34·50	2·8
Green Parakeet*	5	9·50 ± 1·325	3·61 ± 0·376	118	36·60	10·1
Pigeon*	20	8·48 ± 1·679	3·84 ± 0·468	300	31·00	25·0
Common Myna	6	6·22 ± 1·034	2·86 ± 0·226	112	37·70	7·0
Cattle Egret*	4	5·22 ± 0·726	2·09 ± 0·142	347	43·20	21·0
Common Babbler	4	3·97 ± 0·169	1·64 ± 0·109	59	40·20	3·5
Crow Pheasant	4	3·41 ± 0·420	1·76 ± 0·072	233	44·00	13·0
Fowl	5	0·47 ± 0·069	0·31 ± 0·013	1300	64·00	47·0
Bat*	5	9·60 ± 0·990	3·87 ± 0·568	20	35·80	1·8

* Pectoralis major muscles of these birds are of mixed type.

† The values given in these columns are taken from George and Naik (1959b), since the values obtained fairly agree with these authors.

‡ Diameter of the red muscle fibres except for fowl where it is for the light fibre.

The variations in the weight of the pectoralis major muscle also showed some variations in relation to the variations in the body weight in most of the cases. The pectoral muscles of the rosy pastor, sparrow, bat, parakeet and pigeon showed higher concentrations of SDH than that of the myna, egret, babbler and crow pheasant, whereas the pectoral muscles of the fowl showed the least concentration. The results obtained indicate a reciprocal relationship between enzyme activity and body weight, fibre diameter and weight of the pectoralis major muscle within the limits of the animal samples studied. Statistical evaluation, after converting all the values for body weight, weight of the pectoralis major muscle, fibre diameter and SDH activity to their logarithms, results in a regression equation

$$\log X = 6{\cdot}8117 - 3{\cdot}6395 \log D - 0{\cdot}1021 \log B - 0{\cdot}1208 \log P \qquad (1)$$

where $\log X$ is the logarithm of the SDH activity of the pectoralis major muscle,

log D the logarithm of the diameter in μ, log B the logarithm of body weight in g and log P the logarithm of the weight of the pectoralis major muscle in g.

From the regression equation (1) it appears that the regression coefficients of log B and log P are nearly the same and also the correlation coefficient between log B and log P is 0·97. Taking this fact into consideration any one variable can be omitted, say log P, then the regression equation obtained will be

$$\log X = 6{\cdot}5607 - 3{\cdot}4171 \log D - 0{\cdot}2051 \log B \qquad (2)$$

TABLE 2—SEASONAL VARIATIONS IN THE BODY WEIGHT AND THE LEVELS OF SUCCINIC DEHYDROGENASE ACTIVITY OF THE PECTORALIS MAJOR MUSCLE OF ROSY PASTOR

Month	SDH activity in μg formazan/mg dry muscle/30 min	Body weight in g
November	7·7	58
January	9·0	63
February	11·3	68
March	12·7	73
April	16·8	90
Average	11·5	70

The expected values by the equations 1 and 2 also tally with the experimental values. Hence the SDH activity of the pectoralis major muscles of the birds and the bat studied can be correlated in terms of the body weight, fibre diameter and weight of the pectoralis major muscle as shown in the regression equations (1) and (2). Apart from this, the SDH level was found to be significantly increased in the pectoralis major muscle of rosy pastor (*Pastor roseus*) just prior to migration (Table 2).

DISCUSSION

George & Jyoti (1955) and George & Naik (1957, 1959a) recorded some histological and cytochemical observations on the breast muscle of a few birds and a bat and suggested the possibility of some relationship between the structure of the muscle and its function. The reduction in the amount of SDH with increase in fibre diameter has been noted in the mixed muscle of the rat (Nachmias & Padykula, 1958), the bat (George, Susheela & Scaria, 1958) and certain muscles of reptiles (Shah, unpublished). Similar observations have been made by George & Bhakthan (1960a, 1960b) on the concentration of lipase in the flight muscles of certain butterflies and moths as revealed by histochemical as well as biochemical studies. Same holds true in the case of the pectoralis major muscle of different birds when the SDH content is compared in terms of the fibre diameter of the individual muscle.

George & Naik (1959b) recorded the fibre diameter of a few representative types of birds and suggested that in birds with similar flying habits, the mean

diameter of the fibres in the pectoralis major muscle varies with the size of the muscle and also that the rate of increase in the diameter of the fibres/g weight of the muscles differs in birds with different flying habits. Nair (1952) studied the chemical composition of the pectoral muscles of some Indian birds and reported a higher concentration of iron in the good fliers among the birds he studied whereas the lowest iron content was reported in the case of the domestic fowl thereby suggesting a high concentration of myoglobin in the pectoral muscles and consequently higher respiratory metabolism in the good fliers than in the poor or non-fliers. This is also indicated by the SDH levels in the breast muscles of different birds.

A considerably higher concentration of SDH in the pectoralis major muscle than that in the pectoralis minor suggests that the former muscle is metabolically much more active and highly evolved. The pectoralis major muscle of the fowl, which is practically a non-flier, does not show a considerable increase in the concentration of SDH over that of its minor muscle. This may be expected in such a case in which the activity of two muscles is more or less equally restricted.

The various structural and functional features of an organism are known to vary with body size (Adolf, 1949; Fried & Tipton, 1953). The decrease in the respiratory metabolic intensity with increase in body size is reflected in a decrease of the respiratory intensity of several homologous tissues of mammals (Krebs, 1950). There is then an inverse correlation of both the whole body and the tissue metabolic intensity and body size among different species, although intraspecific comparisons do not always demonstrate this relationship (Bertalanffy & Prozynski, 1951; Kunkel *et al.*, 1956).

As is obvious from the results and the regression equations obtained in the present study such a relationship holds good in the case of the birds as well but it should be mentioned that though a reciprocal relationship between the enzyme activity and the other factors studied exist, there can be no definite generalization since there could be exceptions to such relationships which may be produced by the interplay of other factors and are difficult to explain. For example, in the case of the goose which is a poor flier and is as big as a fowl if not bigger, the pectoralis major muscle is composed predominantly of the narrow red fibres. Apart from this, the SDH activity of the breast muscles of birds also depends on the mode of flight.

It is of interest to note that in the case of the rosy pastor which is a migratory form, the pectoralis major muscle shows a marked increase in the SDH activity under standard conditions of the experiments, even with considerable increase in body weight just prior to migration (Table 2). This elevation in SDH level might be due to the higher activity of the thyroid just prior to migration since it is known that cytochrome oxidase activity is elevated in thyrotoxicosis (Kunkel and Campbell, 1952). Kunkel *et al.* (1956) suggested that the cytochrome oxidase activity could be altered by hormonal, dietary or physiological treatment and that a decline in the cytochrome oxidase level with increasing body weight represents the result of the similar adaptive phenomenon. This is again a generalization that cannot be

accepted without reservations. At best the suggestion of Fried and Tipton (1953) that "the constant enzymic activity within a single species indicates indirect genetic factors regulating metabolic enzymes rather than hormonal or other direct somatic factors" appears more tenable. It is likely that the hormonal, dietary and physiological treatment may be secondary factors responsible for regulating the various metabolic processes of the different tissues and ultimately of the organism as a whole but the basic organization being directed by the predetermined genetic factors.

In conclusion it may be said that the results obtained in this study show a relationship more or less similar to what has been observed in the homologous tissues of mammals of different sizes. Though no exact mathematical correlation can be derived between the SDH activity of the pectoralis major muscle and the body weight, and fibre diameter and weight of the pectoralis major muscle, among the different birds studied, a reciprocal relationship, to a certain extent, does seem to exist between the SDH activity and the other factors. This suggestion, however, does not rule out the possibility of this enzyme activity being correlated with other factors which might be concerned with the SDH level of this muscle in birds.

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THE CONNECTIONS BETWEEN THE LATERAL GIANT FIBERS OF EARTHWORMS

D. M. WILSON

Department of Zoology, University of California, Los Angeles,
and Department of Zoology, Yale University

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Abstract—1. Intracellular stimulation and recording techniques show electrical connections between the lateral giant fibers of earthworms. In the species studied the connections attenuate electrical signals about three times.

2. Anodal block of one lateral giant fiber does not delay conduction past the block. The bridges between the two fibers appear to conduct rapidly and the unblocked fiber determines conduction velocity for both fibers.

3. Alcohol in the saline bath depresses conduction between the giant fibers and produces variable delay in crossover. Delayed crossings may be limited to particular regions of the nerve cord. Moving, labile sites of spike crossover, resembling quasi-artificial synapses, probably depend upon the demonstrated connections.

THE structure and ordinary function of the giant fiber system of the earthworm have been largely clarified (Bullock, 1945), but a question has remained as to the nature of the connections between the lateral fibers. The evidence for these connections is mainly physiological with only one report of anatomical links (Stough, 1926). Ordinarily, the two fibers have a single threshold, conduct at equal velocity and in synchrony, but occasionally when fatigued dissociate for a few impulses when stimulated at high frequency (Bullock & Turner, 1950). At threshold intensity, stimulation must often fire only the fiber directly under the electrode. The fact that recording leads invariably show simultaneous activity in both fibers indicates a direct axonal connection between the two, which conducts excitation without measurable delay. Rushton (1945) was able to separate the two spikes in time by surgical interference with conduction in one axon. He found just distal to the cut two spikes about 0·8 msec apart and suggested the time difference to be due to a delay in the crossover. During several centimeters of conduction the latency decreased until the two spikes were again synchronous.

Using techniques of previous workers and intracellular recordings I have attempted to decide between the two possibilities of direct or delaying connections. The presence of direct electrical connections which attenuate both spikes and imposed impulses, reducing them to about one-third of their initial voltage, has been demonstrated. It is suggested that ordinarily these connections conduct normal spikes, and because of the short distance no delay is observed, but that under fatigue or poor physiological conditions conduction fails in the crosslinks before it does in the main fibers and the links transmit with a measurable delay.

Lumbricus terrestris was used in most experiments. Specimens of another earthworm, probably *Eisenia foetida*, were used for a minority of the intracellular observations; 4 or 5 cm of ventral nerve cord were dissected free from other tissue, care being taken during the dissection not to break the gut wall. The cord was mounted with the ends over paired silver electrodes for extracellular stimulating and recording. An intermediate region was held down under slight tension in order to minimize movements of the muscular sheath during intracellular recordings. In some preparations the sheath was split or partially removed and the giant fibers exposed. In most experiments the cord was bathed in the earthworm saline of Rushton (see Kao & Grundfest, 1957).

Stimulation and test pulses were provided by Grass S4 stimulators. Intracellular stimuli and recordings were through glass capillaries filled with 3 M KCl and having a resistance of about 10 megohms. For recording, these were connected to Grass P6 DC preamplifiers through the high impedance MEP6 probes.

RESULTS

Electrical connections between the fibers. Micro-electrodes were inserted into each lateral giant fiber in the same segment. Positioning was observed visually. The criteria of penetration were the shift of baseline representing the transmembrane potential and inversion and increase in amplitude of spikes recorded via the pipette but induced and monitored at opposite ends of the cord. Transmembrane potentials were about 60 mV. Spikes had an amplitude of over 65 mV, a rise time of less than 1 msec, and a decline and after-depolarization lasting several msec. The spikes were similar to those of the median giant fiber as reported by Kao & Grundfest (1957) although the potentials recorded were somewhat smaller. Fig. 1 shows spikes recorded simultaneously in the two lateral fibers.

Under conditions such that large synchronous spikes are recorded from electrodes in each lateral fiber a pulse of either polarity delivered through one electrode may be recorded at the contralateral one. The recorded pulse may reach an amplitude of 5 to 7 mV of depolarization before threshold is reached on the stimulated side. Spike threshold was determined to be about 15 to 20 mV when electrodes are close together in the same fiber. The attenuation between fibers is thus about three times. No rectification was observed when stimulus polarity was reversed.

When the recording electrode was withdrawn from the giant fiber the recorded pulse abruptly declined to zero, except for on-off artefacts, as soon as the tip was away from the membrane though it was still in the cord and at about the same distance from the stimulating electrode. When the same experiment was tried with one electrode in the median giant fiber and the other in a lateral fiber no communicated effects were observed. Kao & Grundfest (1957) also found no effect in the median fiber during spike conduction in the laterals. Both results disagree with Stough's suggestion that anatomical connections also exist between median and lateral fibers in addition to those between the two laterals. The electrical connections between the lateral giant fibers were demonstrated in six healthy

preparations and in several in which spike conduction had failed. No preparation gave a different result.

The course of fatigue of one axon was observed in a preparation with only one lateral fiber penetrated by an electrode. As is observed with extracellular recordings the two spikes became somewhat asynchronous just before total failure of one,

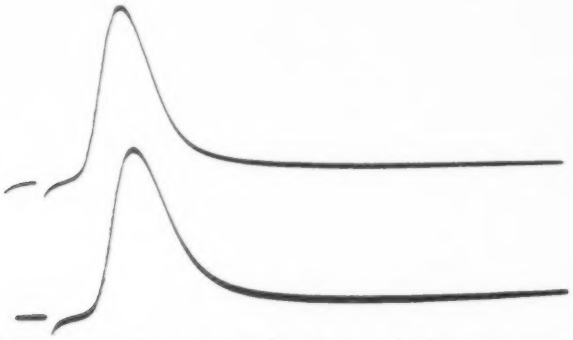

FIG. 1. Simultaneous recording in the two lateral fibers in the same segment. Time from beginning of trace to stimulus artefact is 0·5 msec. Vertical difference between two base lines equals 50 mV. Rise time is about 0·8 msec. Return to resting potential requires several msec. Spike amplitude is about 65 mV.

in this case (Fig. 2(a)) the contralateral one. When the two spikes are synchronous the major part of the recorded potential derives from the penetrated fiber. As the two separate the ipsilateral spike is shown in true proportions, 1 or 2 mV less than

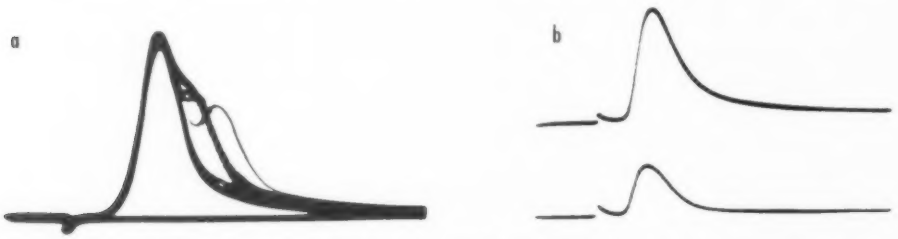

FIG. 2(a). Record from within one fiber during failure of conduction in the contralateral one. Time from beginning of sweep to stimulus artefact is 1·0 msec. Stimulus repeated at 50 times/sec. Total spike amplitude is only 62 mV; this may indicate poor condition of the preparation.

FIG. 2(b). Simultaneous recordings from within the two lateral giant fibers of the same preparation, upper trace same as in 2(a), lower trace of contralateral fiber. Time from beginning of sweep to stimulus artefact is 1·0 msec. Distance between baselines equals 50 mV.

the combined one, and the contralateral spike increases in apparent magnitude, as its latency increases, to a maximum amplitude of about 40 per cent of a full spike. Part of this potential may be contributed by local response which could occur even during the absolute refractory period for spike production, but even so it is induced

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by activity in the other lateral giant fiber since the latency of less than 1 msec does not allow of local reflexes which could produce post-synaptic potentials of the kind described by Kao & Grundfest. The change in contribution of potential from one side to the other with time is probably due either to changing electrical properties of the membrane during passage of the spike or to changing relative refractoriness of a local response.

The axon in which spike conduction has failed might show two kinds of potential, a synchronous passive response to the partner fiber, or a delayed active local potential resulting from the passive one or from activity in another part of the fiber. Immediately following the recording of Fig. 2(a) a second electrode was positioned in the fiber whose spike had failed. Fig. 2(b) shows the simultaneous records from the latter and from the conducting fiber. The inactive fiber records the contralateral spike in attenuated form. Several penetrations in different parts of the segment showed essentially the same spike amplitude in all regions of the fiber. Stimulation through the micro-electrodes also suggested that the one side could not produce spikes. When the normal fiber was stimulated threshold was reached when the blocked fiber showed only 4 to 5 mV depolarization as usual. When a depolarizing voltage was applied to the blocked fiber spike threshold was not reached until the depolarization on the relatively healthy side had reached about 15 mV, the normal threshold for that side, indicating a much larger depolarization at the site of stimulation. The depressed axon was unable to fire when stimulated with pulses much larger than the normal threshold value. Although the potential recorded in the depressed axon may include some local response, this apparently was not propagated from other parts of the axon. Propagation of a potential change of less than spike amplitude could be expected to be slower than the good spike but no asynchrony or modification of waveform is noticeable.

The results directly confirm Stough's suggestion based on anatomy that there are connections between the lateral giant fibers. These could be anastomotic continuities or be divided by membrane similar to the transverse septa of the main axons but in either case they are of low resistance and non-rectifying. The attenuation factor for imposed potential changes of small amplitude is about three times. Spikes in one fiber viewed from the opposite refractory or depressed one are attenuated two to three times as well. Although attenuated, the spike size seen in the opposite silent fiber is so large as to be above threshold for a normal axon.

Single fiber block. By the use of hyperpolarizing pulses through an intracellular electrode, a completely reversible blockage of conduction on one side only was effected, with minimal damage. A blocking pulse, of sufficient duration to overlap with the propagating spikes at the site of electrode penetration, was initiated at the same time as the distant external stimulating pulse. The combined spikes were recorded externally 7 mm beyond the blocking electrode. No effect was observed at the recording site below the intensity of blocking pulse necessary to stop *both* spikes. Both spikes continued to arrive at the same time even though at smaller pulse intensities blocking must have occurred in one fiber alone. Either the impulse crossed over without measurable delay or less than one centimeter of

conduction distance was adequate to re-establish synchrony with the impulse in the other fiber. Neither possibility is expected on the basis of Rushton's observations. The 0·8 msec delay in crossing over could not be demonstrated in this preparation.

These results showing electrical connections and crossover without noticeable delay between fibers led Bullock to suggest (unpublished manuscript) that the bridges ordinarily conduct but that in fatigued or damaged preparations conduction block occurs selectively in the bridges so that one fiber excites the other over a passive bridge with a delay similar to that found in depressed nodes of Ranvier.

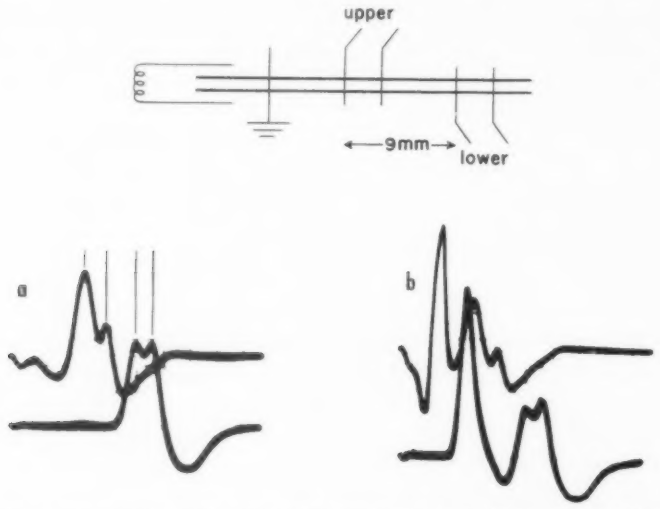

FIG. 3. Externally recorded spikes from the two lateral giant fibers showing dissociation of the action potentials after one fiber has been damaged near the ground electrode. *a* shows the lateral fiber spikes only and *b* both lateral and median. Many superimposed sweeps at 10 times/sec.

Dissociation of function in the two lateral fibers. In *Lumbricus* only, some successful attempts were made to duplicate Rushton's results by his own techniques. The isolated nerve cord was held over a series of silver wires 3 mm apart arranged transversely to the axis of the cord except for two wires at one end which were parallel. The last were used for stimulating selectively only one giant fiber. This arrangement made the separation of thresholds more pronounced since the cathode then lay distinctly nearer one fiber than the other. Appropriate combinations of the other electrodes were used for recordings. Conduction block was produced surgically by puncture of the fiber or cutting of one side of the nerve cord. The results were variable. In some cases no delay was produced but when the other side was damaged in the same segment all conduction ceased, a probable indication that the first attempt at injury had been successful. In other cases asynchrony was observed just distal to the cut, but only 3 mm distant the delay had disappeared. Rarely, a delay persisted throughout centimeters of conduction distance (Fig. 3)

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but decreased gradually (about 0·1 msec/cm) as found by Rushton. The amount of delay was variable but less than 1·0 msec even near the block.

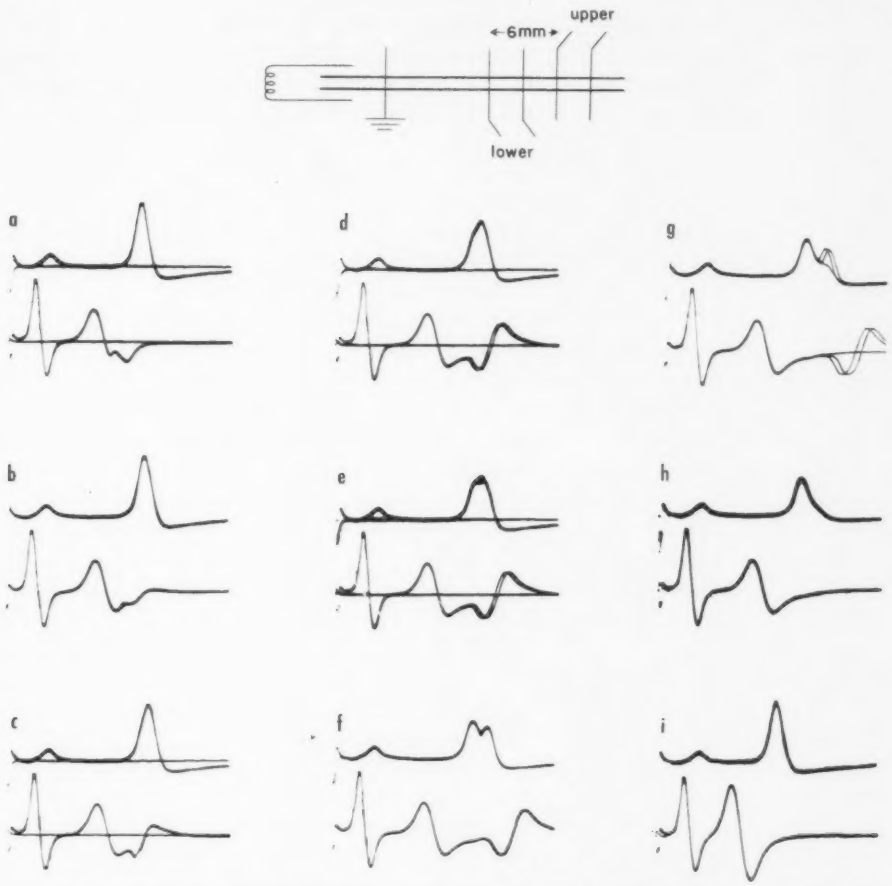

FIG. 4. Externally recorded spikes from the giant fibers of an earthworm cord depressed by alcohol, during stimulation at 10 times/sec. The median fiber spike is blocked between the two electrode pairs. In *a* the lateral fiber spikes are separated by nearly 1 msec at the first pair of electrodes but are synchronous at the second. The latent spike has caught up within 6 mm. The nearly monophasic spikes at the second electrode pair may indicate conduction block between them. In *b* the latent spike has become later and smaller and in *c* even more latent but inverted and larger. It is now arising distal to the first recording pair and conducting toward the stimulated end. In *d* to *f* the delay increases and shows even at the distal electrodes, but as indicated by the spike polarity the locus of cross-over never passes this pair but rather fatigues near them so that in *g* after all-or-none drop out of the spike a small local potential remains, which does not conduct to the proximal electrodes. *h* shows the completely fatigued condition for all spike communication between the fibers and *i* shows the effect of increasing stimulus intensity so that both fibers are fired together. They now behave as independent units. Full sweep equals 10 msec.

Single preparations sometimes fluctuated between synchronous and asynchronous conduction after unilateral damage. Fatigue, drying and overheating tended to cause asynchrony. One preparation which showed a regular delay in crossing was allowed to rest a few minutes in fresh saline after which the delay disappeared. High-frequency stimulation soon resulted in reappearance of a delay between the same electrodes. It is unlikely that the damaged fiber had at any time conducted normally since its membrane had been disrupted over a distance of at least 0·2 mm.

The above results suggested that delayed crossing occurs only under adverse physiological conditions and since it should be easy to produce these a dependable means of obtaining separation of the spikes was sought. The successful search ended with the first method tried, the addition of a few per cent of ethyl alcohol to the bathing solution. 5 per cent alcohol is sufficient to block the fibers completely but if it is added briefly and then washed away with fresh saline it has the effects desired. A progressive dissociation of function occurs as follows: synchronous conduction, delayed conduction (if stimulus is between the thresholds for the two fibers), independent conduction (two thresholds for two spikes which may sum), block of one fiber, block of both fibers. The series of steps reverses as the alcohol is washed out and to some extent the rate of development of each stage and the persistence of a stage can be controlled by the frequency of stimulation. Not all steps can be seen in preparation; some may be passed too rapidly. The effects may be reversed at least three or four times by changing solutions. Most of the results depend on uniform action all along the cord and if there has been prior local damage total block usually occurs at the site of damage.

In several cases when a spike travelled through one fiber without crossing to the other it was reflected from beyond the last electrode presumably by crossing at or near the cut end of the cord. That it was a returning spike could be recognized by the polarity and time of passage at the two electrode pairs used for recording. The reflection was probably not due to an ordinary artificial synapse since it did not excite the median giant fiber which is both closer to the pre-fiber and of lowest threshold for externally applied stimuli. The reflection did not occur if both fibers were excited by the stimulus; it was therefore probably not a return spike in the same fiber. The returning spike had reversed polarity and was of slightly different amplitude. Since reflection could not occur from a lateral to median fiber the cross connections between the two laterals probably played a role.

Reflection of a spike could occur in the middle region of the cord as well. During repetitive firing the locus of the crossing might move along the cord (Fig. 4) in the same manner as in the *quasi-artificial synapses* of Bullock (1953). The movement had the appearance of continuity, but since the sampling was not continuous, continuity is not proven. Fig. 5 shows another case of a moving location of the crossing. In this case the movement was apparently discontinuous and involved two or more preferred loci, all within a length of cord not greater than 5 mm and perhaps as little as 2 mm. The ganglia are 1 to 1·5 mm apart when the cord is stretched to an approximately normal length. Damage to each lateral fiber

in one interganglionic connective, but at opposite ends of the connective abolished conduction in both fibers; damage on either side of a ganglion in some cases did not. These facts suggest that crossing occurs in or near the ganglion. In the case of

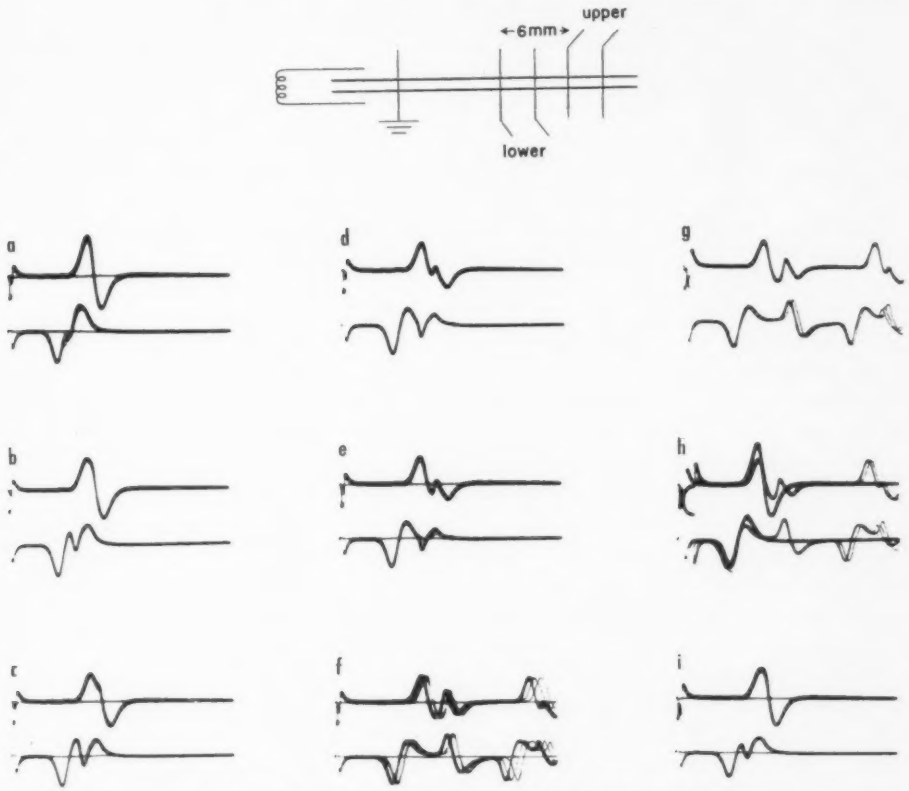

FIG. 5. Externally recorded spikes from the lateral giant fibers of an earthworm nerve cord depressed by alcohol, during stimulation at 20 times/sec. *a* to *d* show development of increased latency of a crossing between the stimulating and first recording electrodes. In *e* the crossing point moves between the first pair on one sweep and in *f* it is located between the two recording pairs; a second reflection somewhere nearer to the recording electrodes causes circus conduction. Conduction times indicate that in *g* the first crossing point has moved even closer to the second recording pair. *h* shows the effect of increasing stimulus strength firing both lateral fibers at once. The summed spikes are nicely biphasic; there are no conduction blocks. *i* shows partial recovery to an earlier stage after a minute of rest. Full sweep equals 10 msec.

block due to damage of alternate fibers on either side of the ganglion, the possibility of damage in the ganglion is not excluded. These results do not prove but are consistent with the notion that there is one bridge per segment located in the ganglion and that the crossing may step from ganglion to ganglion. An unexplored possibility is that the threshold for spike production varies rapidly over small

distances and short times in the alcoholized and fatigued preparations and that apparent continuous movement of crossing results from gradual shifting of the site of spike initiation even while the stimulating current comes from discretely spaced sources.

This preparation showed an additional striking effect when a reflected spike crossed again at the opposite end of the cord. The anodal current apparently had prevented the crossing at the time of the stimulus. The result was a spike which made several round trips, up one fiber and down the other, before fatigue at some spot stopped the circling. Cycling of this kind has been observed also in undissected specimens of the sabellid genera *Schizobranchia* and *Pseudopotamilla* following one-way fatigue of *quasi-artificial synapses*. A single shock elicited a discharge lasting from several to more than a hundred cycles (unpublished observations by the author).

DISCUSSION

Paraphrasing from Bullock (1953) *quasi-artificial synapses* may be defined as labile cross-talks which may occur in unmanipulated preparations between fibers without discernible anatomical connections. A very special feature of these crosses in *Protula* and *Spirographis* appeared to be the moving locus of interaction. The presence of these moving crossings has been shown in some related polychaetes, and, in addition, circus conduction, after unidirectional fatigue of a mid-body crossing, has been identified. The depressed earthworm lateral giant fiber system has here been shown to produce all of these electrically recorded phenomena. The earthworm system has not been tested for certain other features associated with the *quasi-artifical synapses*, namely the effects due to local block of a single active giant fiber. In polychaetes various combinations of ascending and descending spikes are possible when crossing occurs at a conduction block (Bullock, 1953). The only point on which the earthworm system clearly differs from the polychaete one is the described anatomy. Both histological and electrical evidence demonstrate specific connections between the earthworm lateral giant fibers. However, even for the earthworm the anatomy has been reported only once and no graphic evidence supplied. The lack of similar connections in the serpulid and sabellid polychaetes may be doubted until impedance measurements show it as well. The absence of interactions between the adjacent median and lateral giant fibers in the earthworm suggests that an explanation based on external field effects only is not likely for fibers separated by one or two mm of tissue. An interpretation consistent with all the physiological observations is that the *quasi-artificial synapses* are due to electrical continuities which do not conduct spikes between neurons but which allow weaker interactions like those of the depressed earthworm system. It is also possible that they are real synapses or ephapses (in the sense of Grundfest, 1959) but the possibility that they are without anatomical basis seems very slight in the light of the parallel situation in *Lumbricus*. Unless there is positive evidence that the *quasi-artificial synapses* lack an anatomical basis, they should not be held to represent interaction at a distance. Small currents which might be no larger than

those around a propagating axon may influence an already active or near threshold neuron (Terzuolo & Bullock, 1956), but there seems no evidence yet for interactions sufficiently large to cause firing.

The reverberating circuit conduction in the annelid giant fibers provides a striking example of something long talked about in neurology, because it is a really provable case of the cyclic use of the same pathway following a single stimulating input. The phenomenon may in fact be thought of as circus conduction in a single functional unit. Similar activity lasting very few cycles has been seen in the crayfish lateral giant fibers by Furshpan & Potter (1959) who ascribe it to synaptic crossing with one-way injury at some point. It is doubtful that these examples have any more than curiosity value, since they seem to occur only in partially fatigued or unhealthy preparations and thus probably have no significance in the animal's behavior.

Several examples of electrical connections between neurons are now known, each having somewhat different properties. Weak electrical connections which do not conduct spikes and which filter out signals of spike frequency between lobster heart ganglion cells were found by Watanabe (1958). These were shown by Watanabe & Bullock (1960) to spread from cell to cell membrane potential changes which could alter the frequency of continuing activity. In the snail, *Aplysia*, the visceral ganglion contains cells connected so that a spike in one is seen, much attenuated but with similar time course, in another (Tauc, 1959). No functional meaning is known. In the central nervous system of fishes Bennett (1960) and Bennett, Crain, & Grundfest (1959) find low resistance bridges which are the pathways for subthreshold influences having the effect that activity in a group of neurons tends to be synchronous. The occurrence of similar bridges extends to the periphery in lobsters (Reuben, 1960) where connections of unknown function between muscle fibers are found. Connections with a small attenuation have been found between the lateral giant fibers of crayfish (Watanabe, unpublished). This list will no doubt grow both as to kinds and parts of animals involved and degrees of interactions found.

The connections in the earthworm giant fiber system must ordinarily have no integrative action other than that of insuring that both sides always fire in synchrony. In the depressed state the system provides a model for other possible kinds of interaction only a few of which were explored here. On the basis of evidence so far available no decision can be made as to whether the bridges are true protoplasmic continuities as in a syncytium or whether there is a dividing membrane which has the properties of an unpolarized electrical synapse. Operationally the two categories are indistinguishable since both can give the effect of functional continuity.

The range of connections known already in various preparations with wide variations in attenuation and filtering effects shows that many synaptic properties could be mimicked without synaptic boundaries, and the theoretical possibility of a fully integrative but *continuous* nervous system is not excluded. The extent of continuity in real nervous systems and the influence it has on behaviour is yet to be discovered but the possibility that it is important can no longer be ignored.

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TRANSPORT OF MONOSACCHARIDES BY INTESTINAL SEGMENTS OF THE PAINTED TURTLE, *CHRYSEMYS PICTA*

Sr. ALICE M. FOX

Department of Biology, St. Louis University, St. Louis, Missouri*

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Abstract—1. Active transport of some monosaccharides by intestinal segments of the turtle *Chrysemys picta* was measured *in vitro*.

2. Uptake of D-glucose was directly related to the initial concentration in the surrounding fluid.

3. Rate of transport was least at 2°C and greatest (0·46 μM/h/g tissue) at 20–30°C.

4. Segments taken from the upper portion of the tract transported D-glucose at a faster rate than segments from the lower part.

5. D-galactose was transported against a concentration gradient.

6. Mucosal uptake of D-fructose took place, but its translocation to the serosal side was not observed.

7. D-xylose was not transported against a gradient by these preparations.

INTRODUCTION

TRANSPORT of substances against an apparent concentration gradient has been shown to take place across the mucosal epithelium of mammals (Fisher & Parsons, 1950; Crane & Mandelstam, 1960). In contrast to the volume of literature relating to sugar transport in mammalian species, few studies have been made on this process as it occurs in the cold-blooded vertebrates (Csaky & Fernald, 1960; Fox & Musacchia, 1960). The present study deals with the active absorption of monosaccharides by small intestinal segments of the painted turtle, *Chrysemys picta*.

In vitro techniques (Fisher & Parsons, 1949; Wilson & Wiseman, 1954; Crane & Wilson, 1958) have proved useful in the elucidation of transport phenomena. It is recognized that *in vitro* observations cannot be applied without question to the process as it goes on in the intact animal. It is difficult, however, to study absorption against a concentration difference except in an *in vitro* system, since homeostatic mechanisms usually operate to lower the blood-sugar level below that of the gastrointestinal lumen. It is possible to raise the level of blood sugar by injection, and this has been done by some workers (Barany & Sperber, 1939; Campbell & Davson, 1948).

The prime purpose of these studies was to investigate movement of sugar from the mucosal to the serosal side of turtle intestinal segments against an apparent

* Present address: Dept. of Biology, Clarke College, Dubuque, Iowa.

concentration gradient. A second aim was examination of the transport process to ascertain the effects of such variables as (1) the type of sugar presented, (2) initial concentration of the sugar, (3) temperature, and (4) the point along the intestinal tract from which the segment was taken. The third purpose was to observe the transport process in segments taken from turtles in a state of induced cold torpor and to compare these data with those for the active animals.

MATERIALS AND METHODS

Preparation of tissue

The painted turtle, *Chrysemys picta*, was used. Male animals of 5–6 in. shell length were obtained from the Lemberger Biological Supply House in Oshkosh, Wisconsin. One group of animals was maintained at room temperature; a second group was kept in the cold (2–4°C). The steps in preparation of segments of intestine, their incubation in a water bath, and the taking of samples were essentially the same in each experiment. The spinal cord of each animal was cut at the first cervical vertebra, the plastron removed, and segments of intestine excised. For most experiments the segments were taken by severing the gut at a point 1–2 cm posterior to the pancreas.

The method for preparation of intestinal segments *in vitro* was that described by Crane & Wilson (1958) which makes use of everted sacs. One end of each 5–6 cm segment was ligated and the other attached to the tip of a short pipette. The preparation was immersed in 10 ml of the selected fluid in a centrifuge tube. A known volume of the same solution was pipetted into the "serosal lumen" through the upper end of the pipette.

The intestinal preparations were incubated in a ten-gallon, controlled temperature water bath, and each tube was aerated. When temperatures below ambient were required a refrigeration unit was used.

Procedure for taking samples

A 10 min period for equilibration was followed by an incubation period of 60 min. The first samples were taken 30 min after the end of the equilibration period. With a narrow-tip pipette, 0·5 ml of mucosal and serosal fluids were transferred to graduated centrifuge tubes. At the end of 60 min the segments were removed from the water bath, blotted, and one end severed to allow the residual fluid to run into the tube. In this way the total volume of fluid remaining in the sac could be measured. Since this rarely deviated more than 0·1 ml from the predicted volume, it may be concluded that there was little net water movement in either direction.

Chemical compounds

Monosaccharides of the D-configuration were used, e.g. D-glucose, D-galactose, D-fructose, and D-xylose. Stock solutions were prepared in benzoic acid and aliquots mixed with a Krebs-Ringer-bicarbonate solution as required. The buffer formula (Umbreit *et al.*, 1957) was modified to include 0·7 per cent NaCl for use with reptilian tissue.

Chemical analyses

For the determination of reducing sugars the Nelson–Somogyi method (Nelson, 1944) was used. Samples were analyzed for fructose by the method of Roe *et al.* (1949), and for xylose by an orcinol method according to Brown (1946).

Calculations

Changes in total amount of sugar present in the serosal and mucosal fluids at the end of the experimental period are expressed in micromoles (μM) lost or gained per gram of tissue (wet weight) in one hour. To determine the amount of sugar added to or lost from the medium in any interval, the number of milligrams of sugar in the final sample was determined. The difference between initial and post-incubation values was converted to micromoles.

To determine serosal/mucosal (S/M) ratios, the final concentration in mg/100 ml of the serosal fluid was divided by the final concentration in mg/100 ml of the mucosal fluid.

RESULTS

Transport of D-*glucose*

Effect of concentration. Five concentrations of D-glucose were used to test the effect of concentration on absorption rate: 18 mg/100 ml (1×10^{-3} M), 9·0 mg/100 ml (5×10^{-4} M), 4·5 mg/100 ml ($2{\cdot}5 \times 10^{-4}$ M), 2·2 mg/100 ml ($1{\cdot}25 \times 10^{-4}$ M), and 1·1 mg/100 ml ($6{\cdot}2 \times 10^{-5}$ M). Results of this study are summarized in Table 1. It was observed that at 30° a maximal increase (0·46 μM) in transport of glucose to the serosal side took place when 4·5 mg/100 ml glucose was used. The lowest value for serosal increase was found when an 18·0 mg/100 ml glucose solution was used as the initial concentration on either side of the wall. A significant drop in the concentration of the mucosal fluid from its original level was observed at all concentrations used. The extent of this uptake showed a linear relationship to concentration. S/M ratios at the end of experimental periods indicated that concentration gradients were established across the intestinal wall during incubation (at 30°C) with all concentrations of D-glucose. The steepest gradient was observed when 1·1 mg/100 ml D-glucose was used; it declined when segments were incubated with higher glucose concentrations.

Table 1 includes data on the effect of glucose concentration on the rate of transport by intestinal segments taken from cold-torpid turtles and incubated for one hour at 2°C. Mean values for serosal fluid increase in micromoles of glucose were at a maximum (0·50 μM) when 1·1 mg/100 ml was used as the initial concentration, and the values decreased slightly as higher concentrations were used. The changes in glucose content of the mucosal fluid are in contrast to those observed in segments incubated at 30°C. In the experiments with segments from cold-torpid animals, a rise in total D-glucose in both the mucosal fluid and the serosal fluid was observed at the end of one hour. S/M ratios for segments incubated at 2° were lower than those obtained with active animals at 30°.

Effect of temperature. In experiments designed to demonstrate the effect of various levels of temperature on the absorption of D-glucose by *Chrysemys picta* small intestine, preparations from active and cold-torpid animals were run at 2°, 10°, 15°, 20°, 30°, and 37°C (±1°C). In this series of studies a concentration of 4·5 mg/100 ml D-glucose was used. Table 2 summarizes data compiled from incubation of segments at these temperatures. It is evident that the smallest

TABLE 1—EFFECTS OF CONCENTRATION ON GLUCOSE TRANSPORT BY INTESTINAL SEGMENTS (*in vitro*) OF *Chrysemys picta*. MEAN VALUES AND STANDARD ERROR OF THE MEAN ARE GIVEN*

Glucose mg/100 ml	Micromoles glucose/g wet wt. tissue/hr				Terminal S/M ratio	
	Serosal increase		Mucosal change			
	30°C	2°C	30°C	2°C	30°C	2°C
1·1	0·24 (21) ±0·054	0·50 (6) ±0·055	−0·24 (14) ±0·039	+2·70 (6) ±0·212	11·4 (20) ±2·549	2·5 (6) ±0·300
2·25	0·31 (20) ±0·020	0·44 (6) ±0·097	−0·63 (12) ±0·097	+2·30 (6) ±0·376	7·3 (16) ±1·777	2·1 (6) ±0·288
4·5	0·46 (10) ±0·051	0·38 (19) ±0·152	−1·60 (9) ±0·166	+1·40 (19) ±0·217	6·8 (9) ±1·524	1·6 (21) ±0·060
9·0	0·39 (13) ±0·048	0·31 (8) ±0·039	−2·50 (13) ±0·225	+1·00 (8) ±0·300	3·4 (16) ±0·364	1·3 (8) ±0·064
18·0	0·10 (8) ±0·018	0·12 (9) ±0·079	−4·44 (8) ±0·290	+0·15 (9) ±0·052	1·8 (8) ±0·031	1·1 (9) ±0·100

* In these tables, numbers in parentheses refer to the number of intestinal segments used.

increase of total glucose in the serosal fluid occurred at 2°C; values were relatively constant from 10° to 20°, and a maximal increase occurred at 30°C. Mucosal uptake at different temperatures exhibited a minimum at 2°C, reached a peak at 20°, and remained at or near this value with further rise in temperature. The highest terminal S/M ratio was observed when segments were incubated at 20°C.

Incubation of intestinal segments from cold-torpid animals at various temperatures resulted in a net increase in glucose on the serosal side of the wall, but the extent of the rise for the different temperature values did not vary significantly. At 2°, 10°, and 15°C the concentration of glucose in the mucosal fluid was found to be higher at the end of the run than in the beginning. However, when segments from cold-torpid animals were incubated at 20°, 30°, or 37°, there was uptake of glucose from the mucosal fluid in progressively increasing amounts. At temperatures up to 20°C, terminal S/M ratios were low (1·6–2·8) while at 30° and 37°C, the S/M ratios established by segments from cold-torpid animals were as high as those obtained using segments from active animals.

Effect of segment location. The third portion of this study was an investigation of the effect of location of the segment along the intestinal tract. In these experiments only active animals were used, and incubations were carried out at 30°C using three initial concentrations of D-glucose on both sides of the segment: 4·5 mg/100 ml, 9·0 mg/100 ml, and 18·0 mg/100 ml.

TABLE 2—EFFECTS OF TEMPERATURE ON GLUCOSE TRANSPORT BY INTESTINAL SEGMENTS (*in vitro*) OF *Chrysemys picta*. STARTING GLUCOSE CONCENTRATION WAS 4·5 mg/100 ml. MEAN VALUES AND STANDARD ERROR OF THE MEAN ARE GIVEN

Temp. C water bath	Micromoles glucose/g wet wt tissue/hr				Terminal S/M ratio	
	Serosal increase		Mucosal change			
	Active	Cold torpid	Active	Cold torpid	Active	Cold torpid
2°	0·16 (4) ±0·104	0·38 (19) ±0·152	−0·5 (4) ±0·103	+1·4 (19) ±0·217	2·8 (4) ±0·288	1·6 (21) ±0·060
10°	0·31 (4) ±0·055	0·43 (8) ±0·043	−0·6 (4) ±0·180	+2·0 (8) ±0·855	3·5 (4) ±0·418	1·7 (8) ±0·130
15°	0·30 (4) ±0·077	0·42 (7) ±0·143	−1·0 (4) ±0·289	+1·0 (6) ±0·395	3·4 (4) ±0·773	1·7 (7) ±0·225
20°	0·29 (4) ±0·069	0·36 (8) ±0·075	−1·8 (4) ±0·057	−0·6 (4) ±0·086	10·7 (4) ±0·894	2·8 (4) ±0·294
30°	0·46 (10) ±0·051	0·27 (6) ±0·043	−1·6 (9) ±0·166	−1·4 (3) ±0·583	6·8 (9) ±1·524	7·7 (3) ±3·425
37°	0·21 (7) ±0·028	0·28 (4) ±0·102	−1·5 (7) ±0·160	−1·8 (4) ±0·178	7·6 (5) ±4·640	4·3 (3) ±0·288

The results of this study are summarized in Table 3. Values for corresponding segments from the upper intestine are included to provide ready comparison. These results demonstrate that when 4·5 mg/100 ml D-glucose was used, serosal fluid concentration increase was significantly less ($P = < 0{\cdot}05$) in the lower intestinal segments than in those taken from the proximal portion. Serosal fluid increase was not significantly different in the two regions when segments were incubated with 9·0 or 18·0 mg/100 ml D-glucose. Mucosal uptake by the two portions of the tract show a similar trend at all three concentrations, although mean values for the upper intestine were greater in every instance. Terminal S/M ratios were significantly different for the two regions ($P = < 0{\cdot}05$).

Transport of D-*galactose*

Absorption of four concentrations of D-galactose (2·2, 4·5, 9·0, and 18·0 mg/100 ml) against a concentration gradient by intestinal segments (*in vitro*) of *Chrysemys picta* was investigated. Table 4 shows average values for changes in

total reducing sugar concentrations of serosal and mucosal fluids after incubation with the galactose solutions. Except for the lowest initial concentration (2·2 mg/100 ml) a loss of total reducing sugar on the mucosal side was observed. At all

TABLE 3—GLUCOSE ABSORPTION BY LOWER INTESTINAL SEGMENTS CONTRASTED WITH ABSORPTION BY SEGMENTS FROM UPPER INTESTINE OF *Chrysemys picta* AT 30°C. MEAN VALUES AND STANDARD ERROR OF THE MEAN ARE GIVEN

Glucose mg/100 ml	Micromoles glucose/g wet wt. tissue/hr				Terminal S/M ratio	
	Serosal increase		Mucosal decrease			
	Upper	Lower	Upper	Lower	Upper	Lower
4·5	0·46 (10) ±0·051	0·23 (5) ±0·035	−1·60 (9) ±0·166	−0·84 (5) ±0·038	6·8 (9) ±1·524	1·7 (5) ±0·152
9·0	0·39 (13) ±0·048	0·32 (5) ±0·056	−2·50 (13) ±0·225	−2·10 (5) ±0·461	3·4 (16) ±0·364	1·7 (5) ±0·187
18·0	0·10 (8) ±0·018	0·09 (5) ±0·058	−4·44 (8) ±0·290	−3·30 (5) ±1·240	1·8 (8) ±0·031	1·1 (5) ±0·100

TABLE 4—CHANGE IN CONCENTRATION OF TOTAL REDUCING SUBSTANCES IN SEROSAL AND MUCOSAL FLUIDS OF SEGMENTS INCUBATED WITH D-GALACTOSE. MEAN VALUES AND STANDARD ERROR OF THE MEAN ARE GIVEN

Initial galactose concentration in mg/100 ml	Serosal fluid change in micromoles	Mucosal fluid change in micromoles	S/M ratio
2·25	+0·60 (6) ±0·040	+0·66 (6) ±0·286	6·2 (6) ±0·760
4·5	+0·22 (10) ±0·093	−0·50 (10) ±0·097	3·1 (10) ±0·41
9·0	+0·09 (6) ±0·054	−1·20 (6) ±0·261	1·4 (6) ±0·274
18·0	+0·15 (7) ±0·033	−1·80 (7) ±0·290	1·5 (7) ±0·055

four concentrations there was a net rise in reducing substances in the serosal fluid at the end of one hour. Terminal S/M ratios of greater than one were established.

Transport of D-*fructose*

Three concentrations of D-fructose (4·5, 9·0, and 18·0 mg/100 ml) were placed initially on both sides of the intestinal wall. The results obtained are given in Table 5. Six segments of intestine were incubated with each concentration of

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D-fructose used, and in no case was there any increase in the amount of fructose detected in the serosal fluid at the end of a 60 min run. Analysis of samples showed an uptake of fructose from the mucosal fluid. Terminal S/M values at the end of the incubation period scarcely differed from those at zero time. A few determinations showed the appearance of D-glucose on the serosal side when segments had been incubated with D-fructose.

TABLE 5—TRANSPORT OF FRUCTOSE BY INTESTINAL SEGMENTS OF *Chrysemys picta*; AT 30°C. MEAN VALUES AND STANDARD ERROR OF THE MEAN ARE GIVEN

Initial fructose concentration in mg/100 ml	Serosal fluid change in micromoles	Mucosal fluid change in micromoles	S/M ratio
4·5	0 (8) 0	−0·48 (8) ±0·032	1·1 (8) ±0·051
9·0	0 (6) 0	−1·1 (6) ±0·051	1·0 (6) ±0·055
18·0	0 (6) 0	−1·3 (6) ±0·012	1·0 (6) ±0·012

Transport of D-*xylose*

This portion of the study was designed to investigate whether or not isolated segments of *Chrysemys picta* small intestine were capable of transporting D-xylose at various concentrations against a concentration gradient. Solutions of three different D-xylose concentrations were used: 3·0 mg/100 ml (2×10^{-4} M),

TABLE 6—TRANSPORT OF D-XYLOSE BY INTESTINAL SEGMENTS OF *Chrysemys picta*; AT 30°C. MEAN VALUES AND STANDARD ERROR OF THE MEAN ARE GIVEN

Initial fructose concentration in mg/100 ml	Serosal fluid change in micromoles	Mucosal fluid change in micromoles	S/M ratio
3·0	+0·05 (6) ±0·014	+0·10 (6) ±0·033	1·4 (6) ±0·125
6·0	0 (6) 0	−0·19 (6) ±0·004	1·0 (6) ±0·039
13·0	−0·05 (6) ±0·013	0 (6) 0	1·0 (6) ±0·028

6·0 mg/100 ml (4×10^{-4} M), and 13·0 mg/100 ml (9×10^{-4} M). Results as presented in Table 6 indicate that there was no net transport of the pentose from mucosal to serosal side of the segments at any of the concentrations of xylose used. S/M ratios remained close to 1·0.

DISCUSSION

Transport of D-*glucose*

In the experimental procedures reported here, the movement of sugars in a direction opposed to the thermodynamic tendency was taken to indicate function of a mechanism of active transport. The net movement of sugar was found to be from the mucosal surface to the serosal through the intervening tissues. It was anticipated that accumulation of the carbohydrate beneath the serosal surface would bring about diffusion from those cells into the serosal fluid. Measurement of the increased glucose concentration in this fluid gave one indication that active transport had taken place. The second datum which suggested the presence of active transport was a fall in the glucose level in the fluid bathing the mucosal layer. As the glucose concentration in this fluid dropped to a fraction of its original value, back-diffusion would be expected to take place if a mechanism of passive diffusion were the only one operating.

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Effects of concentration. It was observed that the extent of serosal fluid increase in micromoles of glucose after one hour of incubation at 30° showed a linear relationship to concentration of the test solution up to a maximum. Thus, uptake of glucose by the cells of the mucosal epithelium and its passage to the serosal fluid appears to be influenced by concentration, at least in the range of concentrations studied.

Table 1 demonstrates the establishment of a steep concentration gradient between mucosal and serosal fluids during the course of the one hour incubation period for all starting concentrations except 18·0 mg/100 ml. Since the movement of substances against a concentration gradient is generally accepted as a criterion of active transport (barring excessive streaming of water), it may be concluded that segments of small intestine from *Chrysemys picta* are capable of active transport of D-glucose under these conditions.

The picture of glucose transport in the cold-torpid turtle appears to be complicated by the existence of an endogenous source of glucose, probably glycogen, which leaves the intestinal wall during incubation and passes into both serosal and mucosal fluids. Thus, in these animals, but not in those kept at room temperature, one observes a rise of glucose on the mucosal side of the wall as well as on the serosal side.

Effect of temperature. There have been a number of studies giving evidence for a temperature effect upon active transport of D-glucose. The early work of Ohnell & Hober (1939) contained the observation that low temperatures more or less abolished the factor of special transport and equalized the absorption of "physiological" and "nonphysiological" sugars. Macleod *et al.* (1930) observed a stimulatory effect of higher temperatures upon glucose absorption in contrast to the diffusion of xylose across the intestinal wall of the rat. It was shown that a temperature of zero for one hour did not destroy the viability of excised segments of intestine nor prevent them from actively transporting glucose when the temperature was raised.

In the light of Q_{10} values found for certain biological processes, including transport (Crane *et al.*, 1957), the temperature effect on this reptilian system was less than one would expect. Q_{10} values for uptake and transport were in a range from 1–3. Optimal conditions for transport activity appeared to be in the 20–30° range, since serosal increase, mucosal uptake, and S/M ratio all show maxima at these temperatures. The "plateau" of values from 10–20° is of interest since it appears to establish a physiological limit in the turtle. In addition, it is of note that temperatures of 10°C or above appear to be characteristic of behavioral activation and hibernal viability in this animal (Sexton, 1959; Musacchia, 1959).

The effect of temperature upon the transport mechanism is strikingly illustrated in the case of the incubation of segments of intestine from cold-torpid animals. The increase in serosal concentration is quite uniform over the entire range of temperature variation, but the concentration changes in the mucosal fluid showed an interesting alteration. In the lower portion of the range (2–15°C) glucose left the wall and moved into the mucosal fluid more rapidly than it was taken up. At 20° and higher, mucosal uptake was observed, and it increased with a rise in temperature. S/M ratios showed a regular increase as the temperature was raised. These data would seem to indicate that, while active transport may be minimal in the cold-torpid condition, the tissues of these animals retain the capacity to transport glucose by an active process even when they have been in the cold for over two weeks without food.

Effect of location of segment. S/M ratios established by the segments from the "colon" of the turtle show a significant difference from S/M ratios of the upper intestinal segments. This fact indicates that the D-glucose transport rate is less in segments from the lower portion of the turtle intestine than in the upper region. This conclusion is in agreement with observations made of absorption from the lower part of the intestinal tract in many mammalian species. A number of workers have shown that the rate of glucose transfer by proximal jejunum is greater than that of more distal parts of the small intestine (Magee, 1930; Wood, 1944; Fisher & Parsons, 1950; Korelitz & Frank, 1959).

Transport of D-*galactose*

A number of studies on absorption in mammalian and avian species have shown that D-galactose is transported against a concentration gradient by segments of small intestine (Wilson & Vincent, 1955; Fisher & Parsons, 1953; Landau & Wilson, 1959). Galactose absorption has also been demonstrated in cold-blooded animals: in frogs by Minibeck (1939), Westenbrink & Gratama (1937), and Cordier & Worbe (1954); in the marine fish *Scorpaena porcus* by Cordier & Chanel (1951, 1953). Work on the poikilotherms, however, has been confined to *in vivo* studies of absorption, and active transport has not been specified as the mechanism involved. The studies reported here were designed to demonstrate movement against a concentration gradient.

It may be concluded from the present investigation that intestinal segments of *Chrysemys picta* are capable of transporting D-galactose against a concentration

gradient. At concentrations above 4·5 mg/100 ml serosal increase tends to diminish, while uptake from the mucosal surface increases linearly with initial concentration. S/M ratios were above 1·0, but not as high as those established during glucose transport.

Transport of D-*fructose*

Studies of the absorption of D-fructose by mammalian intestinal preparations have yielded results which have been variously interpreted. Though Verzar (1935) stated that fructose absorption in rats was inhibited by iodoacetic acid, Riklis & Quastel (1958) found that phlorizin had little effect on the process as it occurred in guinea-pig intestine. In general, it has been observed that fructose is absorbed *in vivo* at a faster rate than the sugars which are taken up by diffusion alone, yet its passage is less rapid than that of glucose which is known to be actively transported (Cori, 1925; Westenbrink, 1936; Csaky & Humm, 1956).

D-fructose absorption against a concentration gradient was investigated by Wilson & Vincent (1955) using the golden hamster. They concluded that intestinal segments of this animal did not transport D-fructose actively, and the intake fructose molecule was moved by passive diffusion across the intestinal wall. The same conclusion was reached by Darlington & Quastel (1953) working with guinea-pig intestine. Absorption *in vivo* of D-fructose in cold-blooded animals was studied by Cordier and co-workers (1951, 1953, 1954), but to our knowledge no studies have been designed to demonstrate transport of fructose against a concentration gradient by intestinal segments from poikilotherms.

It was proposed by Verzar & McDougall (1936) that the relatively low absorption rate of fructose was due to a partial transformation of fructose to glucose in the intestinal wall, and that the intact molecule of the ketose was absorbed by diffusion alone. Work by Kiyasu & Chaikoff (1957) using ^{14}C-fructose gave further evidence for the truth of this explanation.

In the light of the work on mammalian preparations, some interpretation may be made of our observations relating to intestinal absorption of D-fructose by *Chrysemys picta*. There is evidence of uptake of fructose from the mucosal fluid at all concentrations studied. The establishment of near-equal concentrations of fructose across the intestinal wall suggests that passive diffusion is operating. It is possible that some conversion of fructose into glucose took place, as was indicated by the appearance of glucose in the serosal fluid. The techniques used, however, did not permit a distinction to be made between glucose diffusion from a glycogen deposit in the wall and glucose transport as a result of conversion of fructose to glucose.

Transport of D-*xylose*

A number of studies of the absorption of xylose by mammalian preparations (Auchinachie *et al.*, 1930; Wilbrandt & Laszt, 1933; Brien *et al.*, 1952; Fullerton & Parsons, 1956) indicate that absorption of the pentose takes place and that its mechanism is that of passive diffusion.

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This study was designed to demonstrate whether or not isolated segments of *Chrysemys picta* small intestine were capable of transporting D-xylose at various concentrations against a concentration gradient. It may be concluded that at 30°C segments *in vitro* do not actively transport D-xylose. Terminal S/M ratios indicated that a passive diffusion mechanism was operating. This conclusion is in agreement with others (Wilson & Crane, 1958) who included D-xylose with other five-carbon sugars not actively transported by segments of hamster intestine.

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THE EFFECTS OF ESERINE ON SODIUM REGULATION IN CRAYFISH*

F. I. KAMEMOTO

Department of Zoology, University of Missouri, Columbia, Missouri

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Abstract—1. The concentration of sodium in the blood of the crayfish, *Orconectes virilis* Hagen, is decreased from a mean value of 220 m. equiv/l. to 196 m. equiv/l. over a 24-hr period with the injection of eserine. This is accompanied by an increase in the total outflow of sodium into the medium.

2. The concentration of sodium in the urine is drastically increased 3 hr after the injection of eserine with a partial return to normal at 12 hr.

3. Control injections with water and sucrose cause no change in the concentration of sodium in the urine.

4. Eserine injections caused an increase in the total osmotic concentration as determined by the freezing point method.

5. The data are discussed in light of evidence that the crayfish kidney is a filtration-reabsorption kidney, and that cholinesterase, found in both kidney and gills of crayfish, is involved in the regulation of sodium.

INTRODUCTION

SINCE the studies of Kirschner (1953) reporting the association of cholinesterase with sodium transport across the isolated frog skin, considerable interest has been focused on the possible role of cholinesterase in active transport. This association has been further studied by Koch (1954) in the gill of *Eriocheir sinensis* and the anal papilla of *Chironomus plumosus* larva, Van der Kloot (1956) in sartorius muscle of the frog, Holland and Klein (1956) in red blood cells and Koblick (1958) in frog skin. These studies were all made on isolated animal tissues. It is then of interest to examine the role of cholinesterase in sodium regulation in intact animals.

The crayfish *Orconectes virilis* Hagen was selected for studies on the influence of the reversible cholinesterase inhibitor, eserine, on the regulation of sodium in the body fluid. The crayfish is an ideal animal for such studies since it inhabits fresh waters, its blood being highly hypertonic to the medium. It produces a urine in a single pair of filtration-reabsorption kidney tubules (Martin, 1957; Riege & Kirschner, 1960). This urine is extremely hypotonic to the blood and can be readily collected under normal conditions. In this paper, the effects of eserine on blood and urine sodium concentrations of crayfish will be discussed.

MATERIALS AND METHODS

Orconectes virilis Hagen was obtained from E. G. Steinhilber and Co., Oshkosh, Wisconsin, during November and December, 1960. Animals were held in tap

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water, in aquaria, in a constant room temperature at 16°C for 7–14 days before being used in the experiments. All experiments were carried out at this temperature.

The animals used ranged from 20 to 35 g in body weight. Blood was collected by inserting a drawn capillary through the membrane at the base of the last pereiopod and draining onto a piece of parafilm. A fifty μg sample of blood was diluted with an equal volume of deionized water, mixed vigorously to coagulate the blood and centrifuged. The supernatant was then analyzed for sodium with the Coleman Flame Photometer. Larger volumes of blood were collected and treated in a similar manner for animals used in the study of blood total osmotic concentrations. The supernatant was diluted, and its freezing point determined with a Fisk Cryoscope. The freezing point data are presented in terms of standard NaCl solutions.

Urine was collected according to the method outlined by Riegel and Kirschner (1960). Except for a few cases, urine was readily obtained from the animals by the use of this method. Urine sodium was analyzed directly without further treatment of the urine, a measured sample being diluted and analyzed in the flame photometer.

Injections of test solutions were made directly into the hemocoel through the ventral abdominal muscles, directing the syringe needle anteriorly into the thoracic region. This prevented any visible outflow of fluids from the crayfish as the needle was withdrawn. The details of each experiment will be presented with the results of this study.

TABLE 1—EFFECTS OF ESERINE ON BLOOD SODIUM AND TOTAL OSMOTIC CONCENTRATIONS IN *Orconectes virilis* HAGEN

Hours after injection (20 μg of 0·01 M eserine)	No. of animals	Blood sodium (m. equiv/l.)	No. of animals	Osmotic conc. expressed as (m. equiv NaCl/l.)
0 Control	18	220·5 ± 7·3	8	254·0 ± 11·9
1	18	217·1 ± 9·1	7	258·9 ± 13·6
3	18	205·1 ± 7·6	8	267·5 ± 17·7
6	17	203·0 ± 8·7	8	279·5 ± 11·8
9	10	203·0 ± 8·6	—	—
12	17	198·1 ± 11·5	8	282·5 ± 11·5
24	16	196·2 ± 15·1	8	304·0 ± 19·4

RESULTS

Effect of eserine on blood sodium and total osmotic concentrations

Animals in tap water were injected with 20 μg of 0·01 M eserine. At this dosage (approximately 2×10^{-4} M in the blood for a 30 g animal), the animals react with violent contractions of the abdominal muscles, followed by paralysis, with visible activity remaining only in the mouth appendages. The animals remain in this condition for several hours, followed by an apparently complete recovery in 12–24 hr after injection. Only in a few cases did the animals die following this dosage of inhibitor.

Blood was collected at time intervals of 1, 3, 6, 9, 12, and 24 hr post injection and analyzed for sodium and total osmotic concentrations. The results are presented in Table 1. The mean blood sodium level in the control group receiving 20 μg of deionized water per animal was 220·5 m. equiv/l. blood. This mean value is equal to that for untreated control animals. The sodium level decreased to 205·1 m. equiv/l. within 3 hr after injection of eserine with a further gradual drop

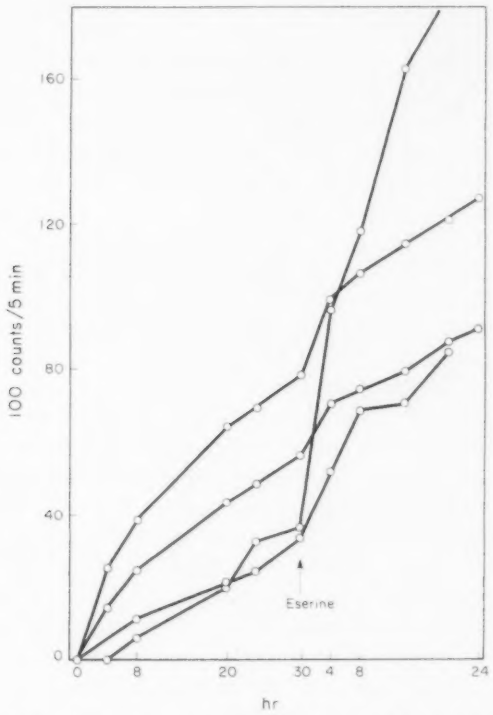

FIG. 1. Total outflow of Na^{22} at time intervals before and after injections of 20 μg of 0·01 M eserine.

to a mean value of 196·2 m. equiv/l. at the end of 24 hr. In some cases, however, there was a return to normal values after 24 hr as indicated by the wide range in the standard deviations. The total osmotic concentrations, on the other hand, increased with the injection of eserine, and continued to increase over the 24 hr study period.

Effect of eserine on total sodium outflow

The fact that the injection of eserine into the hemocoel of crayfish resulted in the lowering of the concentration of sodium in the blood suggests that eserine causes an increase in the total outflow of sodium of crayfish kept in tap water. To

determine if, in fact, there is an increase in total outflow of sodium with eserine injections, four crayfish were injected with 0·5 ml of 0·3 M NaCl labeled with Na^{22}. The amount of Na^{22} in the medium was then determined by taking samples of the medium at various intervals and counting by standard methods. Thirty hours after the injection of Na^{22} added to NaCl, the animals were injected with eserine. The results are presented in Fig. 1; with the injection of eserine, there is a sudden increase in the appearance of Na^{22} in the medium in all four crayfish studied.

Effect of eserine on urine sodium

The injection of eserine into the hemocoel of crayfish caused a decrease in the concentration of sodium in the blood accompanied by an increased outflow of sodium into the medium. To determine whether these were the result of an increased loss of sodium in the urine, the concentration of sodium in urine was determined for animals injected with eserine. Urine was collected from individual animals and sodium levels determined for a period of 1–2 days to determine the normal level of urine sodium in the particular animal under normal conditions. Urine was collected just prior to the injections of test solutions. The first urine collection after injections was made at three hours because it had been noted in the first experiment that the greatest drop in blood sodium occurred in the first 3 hr. No further collection was made until 12 hr after injection because of the difficulty of urine collection at closer time intervals. Control animals were run in two groups, the first receiving 20 μg of 0·01 M sucrose and the second, an equal volume of deionized water. Sucrose was used to eliminate the effects of solutes introduced into the animal on the formation of urine. The experimental animals received the same dose of eserine as in the previous experiments.

The results are presented in Table 2. Nine animals were injected with eserine. There is very little fluctuation in the concentrations of sodium in the urine when normal urine samples were collected at 24-hr intervals. With the injection of eserine, however, there is a sharp rise in the concentration of sodium in urine in 3 hr with the highest value of 175 m. equiv/l. urine. There is a drop of urine sodium in 12 hr with almost a complete recovery in three of the animals in 24 hr.

In experiments with animals injected with water and sucrose, there is no definite change in urine sodium levels. Only in one water-injected animal is there a noticeable increase in the concentrations of sodium in the urine.

DISCUSSION

The functional mechanism of the crayfish kidney has been the subject of some discussion. Maluf (1941a, b) suggests that the kidney of *Cambarus clarkii* forms urine by active secretion of salts and water. Martin (1957) presents evidence that Maluf's data could be interpreted to show filtration into the end sac, followed by reabsorption of salts from the tubule. Riegel and Kirschner (1960) conclude from studies on inulin and glucose excretion that filtration does occur in this organ accompanied by the reabsorption of salts. Peters' data (1935) on the chloride content of fluids from different parts of the kidney of *Potamobius fluviatilis* suggest

a reabsorption of the ion from the primary urine as it passes distally in the tubule to the bladder. Assuming then that the crayfish kidney is in fact a filtration-reabsorption kidney, and that the enzyme cholinesterase is associated with active uptake of sodium (Kirschner, 1953; Koch, 1954; Van der Kloot, 1956), then one

TABLE 2.—CHANGES IN URINE SODIUM IN ANIMALS INJECTED WITH 20 μg OF 0·01 M ESERINE (E), 0·01 M SUCROSE (S) AND DEIONIZED WATER (W)

Animal No.	Urine sodium in m. equiv/l.					
	Time in hours					
	−48	−24	0	3	12	24
E–1	53	85	73	127	64	66
E–2	19	28	23	175	146	118
E–3	40	34	38	160	144	140
E–4	*	54	53	160	90	90
E–5	12	18	16	143	57	40
E–6	10	17	20	133	64	*
E–7	22	13	9	21	38	5
E–8	*	27	23	133	67	30
E–9	*	15	31	155	160	*
S–1	29	23	20	27	28	43
S–2	*	14	13	18	14	12
S–3	*	15	12	11	14	14
S–4	*	20	15	30	14	19
S–5	30	27	36	31	20	23
S–6	57	35	34	31	20	20
S–7	32	*	29	32	12	32
S–8	13	14	20	21	13	19
W–1	21	24	45	90	65	31
W–2	39	27	15	19	16	13
W–3	34	53	36	36	32	*
W–4	10	17	25	25	21	20
W–5	40	21	27	20	15	12
W–6	15	12	14	30	17	17
W–7	*	14	16	30	15	11
W–8	*	65	79	82	52	*

* Sample not obtainable.

might expect that this enzyme is present in the kidney. There is no published observation to this effect. Preliminary observations in this laboratory, however, indicate the presence of cholinesterase in the kidney of crayfish and, in fact, also in the gills. Studies are being conducted on histochemical localization and chemical characterization of the enzyme in the kidney and gills of crayfish. The results of these studies will be presented in a subsequent paper.

Koch (1954) demonstrated the presence of cholinesterase in the gills of *Eriocheir sinensis* and presented evidence for the localization of the enzyme in the

gill epithelium itself. He also demonstrated the active uptake of sodium by the isolated gills. Active uptake of sodium by the gills is inhibited with eserine, the inhibitory action of eserine being entirely reversible after washing with water. Although it is clear that crayfish do absorb sodium from hypotonic media (Shaw, 1959, 1960a, b), the actual site of sodium uptake has as yet not been established.

The results presented in this paper suggest that the injection of eserine into the hemocoel of crayfish causes a drop in the blood sodium levels (Table 1) and an increase in the urine sodium levels (Table 2). The drop in blood sodium is most probably due to the increased outflow of sodium into the medium as indicated by studies with Na^{22}. Bryan (1960b) noted that crayfish exposed to a 3 m. mole/l. solution of eserine appeared to lose sodium and that the animals were dead after 3-hr exposure. The increased outflow of sodium with the injection of eserine is probably due to the passage of sodium by the kidneys as indicated by the increased concentrations of sodium in the urine. Bryan (1960a) calculates that only about 6 per cent of the total sodium outflow is due to the kidneys in animals held in tap water. In salt-loaded animals, however, up to 70 per cent of the total sodium outflow is from the kidneys (Bryan, 1960c). The animals used in the radiosodium study were injected with 0·5 ml of 0·3 M NaCl solution. The mean concentration of sodium in blood was found to be 220 m. equiv/l. blood. If we take 1·118 as the specific gravity of crayfish (Bryan, 1960a) and the blood volume of *Cambarus virilis* as 30·5 per cent of the total volume (Prosser & Weinstein, 1950), we can arrive at a blood volume of 8·2 ml for a 30 g crayfish. The injection of the salt solution, then, would raise the blood sodium level for this animal from 220 to 225 m. equiv/l. blood, well within the standard deviation of normal crayfish values. If we assume that *Orconectes virilis* is like *Astacus fluviatilis*, then according to the calculations of Bryan, most of the sodium in these animals is lost through the gills, and the effect of eserine causing an increased outflow of sodium may be in the inhibition of gill cholinesterase causing an increased permeability of the gills to sodium as shown to be the case in *Eriocheir sinensis* by Koch and Evans (1956).

The injection of eserine in crayfish caused an increase in the urine sodium levels (Table 2). This may be due to the inhibition of reabsorption of sodium from the primary urine by the kidney tubule with the inhibition of cholinesterase, and suggests that cholinesterase may be involved in the active reabsorption of sodium from the crayfish kidney.

Perhaps it might be argued that the increased osmotic concentration of the blood caused by the injection of eserine (Table 1) causes the increase in urine sodium to some extent. This argument, however, may be dismissed because the urine sodium levels drop after three hours in most cases while the blood osmotic concentration continues to rise even at 24 hours after the injection of eserine. Riegel and Kirschner (1960) show increases in urine sodium in control animals and suggest that this is associated with rises in blood glucose due to handling. Unfortunately, they report percent increases of sodium concentrations in the urine which prevent comparisons with the present data. I found no increase in urine sodium levels due to handling in animals injected with sucrose and water.

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If the crayfish kidney is in fact a filtration-reabsorption kidney, the results of this study support the idea that a cholinesterase system is involved in the sodium transport mechanism as originally suggested by Kirschner and supported by others. Obviously, further studies are indicated to show the relationship of cholinesterase and sodium regulation in the crayfish.

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FREE AMINO ACIDS IN THE HEMOLYMPH OF THE AMERICAN COCKROACH, *PERIPLANETA AMERICANA* L.*

T. M. STEVENS

Department of Microbiology, Saint Louis University School of Medicine, Saint Louis, Missouri

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Abstract—1. Free amino acids of the hemolymph of the American cockroach, *Periplaneta americana*, were determined quantitatively by the column chromatographic method of Moore, Spackman, and Stein.

2. Sixteen amino acids [aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, methionine (and methionine sulfoxide), isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine, arginine] were present in the high concentrations characteristic of insect hemolymph.

3. Taurine was also present in low concentration. Cystine was consistently absent.

4. Total concentrations were comparable with those of other relatively primitive insects (e.g. walking-stick and dragonfly) but were only one-third to one-half those of holometabolous insects (e.g. silkworm and honeybee).

5. The data suggest that individual and total amino acid concentrations are not closely regulated in this insect.

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INTRODUCTION

A QUANTITATIVE analysis of the free amino acids in hemolymph of the American cockroach, *Periplaneta americana* L., was undertaken because it may provide data useful for the development of a medium in which to grow cells or tissues of this insect. A similar analysis of silkworm hemolymph (G. Wyatt *et al.*, 1956) was the basis for a medium which has been used with success for cultivation of lepidopterous tissues (S. Wyatt, 1956; Grace, 1958).

Studies on insect hemolymphs have shown them to contain free amino acids in exceedingly high concentrations, often 50 to 100 times higher than the concentrations found in man (Bishop *et al.*, 1925; Ussing, 1946; G. Wyatt *et al.*, 1956; Duchâteau & Florkin, 1958). This unique phenomenon has been proposed as a taxonomic characteristic of the class Insecta (Florkin, 1949).

Most of the previous studies on various insects have been based on colorimetric tests specific for particular amino acids, on paper chromatographic methods, and on microbiological assays. In the work reported here the free amino acids were separated by ion exchange column chromatography and measured colorimetrically with ninhydrin. This method has the advantage of determining a large number of amino acids with great accuracy, but requires substantial amounts of hemolymph.

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MATERIALS AND METHODS

Collection of hemolymph

Hemolymph was collected and pooled from adult cockroaches of both sexes over a period of more than a year. The roaches were drawn from two unrelated colonies maintained at Saint Louis and Rutgers Universities. They were bled by cutting off two hind legs. The three or four drops of usually clear hemolymph which exuded from the severed coxae were aspirated through polyethylene tubing into a graduated centrifuge kept in an ice bath. After collection of 1 or 2 ml, the hemolymph was centrifuged at 4°C for 10 min at 1200 to 1500 rev/min to remove cells and the small amount of coagulum which accumulated during collection. The total sediment after centrifugation never exceeded 1 to 5 per cent of the volume. The supernatant was lyophilized.

Deproteinization

Lyophilized samples representing 1 to 2 ml of whole hemolymph were dissolved in distilled water and diluted to 5 ml. The diluted samples were then deproteinized with picric acid according to the technique of Stein and Moore (1954).

Separation of free amino acids

Amino acids were separated on the Amberlite cation exchange columns of Moore, Spackman, & Stein (1958) and concentrations determined by the ninhydrin photometric method of Moore & Stein (1954a). A 150 cm column was used to separate the acid and neutral amino acids and a 15 cm column to separate the basic amino acids.

A sample of the deproteinized hemolymph adjusted to pH 2 and corresponding to 0·25 to 0·5 ml of whole hemolymph was placed on the column and washed in with three 0·3 ml aliquots of pH 2·2 citrate buffer (Moore & Stein, 1954b). For runs during which 1 ml instead of 2 ml samples of effluent were collected (see below), the column was loaded with the equivalent of up to 1 ml of whole hemolymph.

If a peak in the hemolymph chromatogram was displaced to one side of its location in the standard amino acid runs, a subsequent hemolymph run was made with a known amount of the amino acid in question added to determine whether the shift in position was due to some variation in the run or to a compound other than the amino acid suspected.

Identification of unknown and mixed peaks

The amino acid composition of unknown or mixed peaks from the column was determined with the help of paper chromatography. For this purpose the usual procedure of collecting the effluent in 2 ml samples, which were then treated with ninhydrin, was altered. Instead, 1 ml samples were collected and even-numbered tubes treated with ninhydrin to locate the unknown peak precisely. The odd-numbered tubes in the area of the peak were desalted on Dowex 2 columns by the procedure of Dreze, Moore, & Bigwood (1954). The desalted effluent was

concentrated to a volume of 0·1 ml or less and analyzed by ascending chromatography on Whatman No. 1 paper in one or two dimensions. Paper chromatographic methods and solvents were those of Redfield (1953). Known amino acids and derivatives shown by Moore, Spackman, & Stein (1958) and Spackman, Stein, & Moore (1958) to move on the columns in the area in question were chromatographed on paper with the unknown, as were mixtures of the unknown and known substances.

Reproducibility

Replicate runs made with a standard amino acid mixture ranged from 100 ± 5 per cent to $\pm$ 10 per cent recovery as compared with ± 3 per cent reported by Moore, Spackman, & Stein (1958).

Resin

All materials used for column chromatography were as specified by Moore, Spackman & Stein (1958) with the exception that part of the work was done with columns poured with commercially fractionated resin. This resin was supplied by Bio-Rad Laboratories, Richmond, California, as Aminex–M S cation exchange resin, Modified Fraction D.

RESULTS

Table 1 summarizes the results of six runs on the 150 cm Moore–Stein columns. Columns A–E represent five chromatograms on five different pools of hemolymph. Column F consists of duplicate runs on a sixth pool of hemolymph. Both values are included as an indication of the precision of the methods employed. Table 2 lists the concentration of the basic amino acids, lysine, histidine, and arginine, determined on the 15 cm column on three different pools of hemolymph.

From the data it is evident that a high concentration of glycine is characteristic of the American cockroach. Proline, glutamic acid, tyrosine, and histidine are also found in high concentrations as compared with most of the remaining amino acids. The extreme variability of proline and glutamic acid is noteworthy.

In Table 1, serine and threonine are enclosed in quotation marks because the peaks invariably ran together to some extent due to the presence of other substances. When the desalted column eluate containing these peaks was placed on paper, the ninhydrin-positive material was found to be mainly serine and threonine. Small amounts of glutamine and asparagine, as well as traces of three unidentified compounds were also found. No attempt has been made to measure these latter peaks quantitatively. The threonine value includes some glutamine and a trace of an unknown; the serine value includes some asparagine as well as traces of two unknowns. The extent of hydrolysis of glutamine to glutamic acid and asparagine to aspartic acid due to the picric acid method of deproteinization or to the elevated temperature and lowered pH of the column was not determined. The glutamic acid and aspartic acid concentrations as determined here may therefore be somewhat high.

A small unknown peak at the beginning of the chromatogram was identified on paper as taurine. This peak was never quite symmetrical and paper chromatograms showed a trace of another, unidentified, substance.

TABLE 1—ACID AND NEUTRAL FREE AMINO ACIDS OF HEMOLYMPH OF *Periplaneta americana* L. (mg/100 ml)

	St. Louis University colony		Rutgers University colony					Average and standard error*
	A	B	C	D	E	F‡		
Taurine	3·5	6·4	7·9	2·5	—	1·4	—	4·7 ± 1·1
Methionine sulfoxides†	8·1	8·7	13·0	11·7	5·1	13·7	12·8	9·1 ± 1·3
Aspartic acid	0·7	3·4	1·7	1·0	5·6	1·6	1·4	2·3 ± 0·8
"Threonine"	4·7	6·7	8·7	25·6	8·9	8·5	9·2	7·6 ± 0·8
"Serine"	14·4	15·9	9·3		8·2	23·1	22·7	14·1 ± 2·4
Glutamic acid	35·4	39·3	5·5	5·9	50·2	6·1	5·9	23·8 ± 8·2
Proline	16·5	30·1	65·2	42·5	48·6	52·6	52·2	42·5 ± 7·0
Glycine	38·0	47·5	49·4	62·4	57·3	62·5	63·0	52·9 ± 4·0
Alanine	6·6	7·2	5·8	8·0	8·8	7·8	7·3	7·3 ± 0·5
Valine	10·5	9·9	9·0	13·0	9·2	14·3	11·9	10·8 ± 0·7
Methionine	—	2·4	2·4	—	7·4	3·9	5·0	4·2 ± 1·0
Isoleucine	3·4	4·1	5·5	7·5	6·4	6·6	6·9	5·6 ± 0·7
Leucine	4·9	6·4	5·5	5·2	10·7	8·3	7·8	6·8 ± 0·9
Tyrosine	19·6	23·7	20·5	32·8	24·3	30·8	28·7	25·1 ± 2·1
Phenylalanine	4·5	5·8	7·7	9·7	4·6	5·5	5·8	6·3 ± 0·9
Total	172·8	217·5	218·1	227·8	255·3	246·7	240·6	223·1 ± 12·0

* Averages of columns A–F and standard errors of the means.

† Calculated as methionine.

‡ Duplicate runs on a single pool of hemolymph. The average of the paired data in this column was used with the data of columns A–E in calculating the averages shown in the final column.

TABLE 2—BASIC FREE AMINO ACIDS OF HEMOLYMPH OF *Periplaneta americana* L. (mg/100 ml)

	St. Louis University colony	Rutgers University colony		Average and standard error*
Lysine	7·0	12·6	13·2	10·9 ± 2·0
Histidine	17·8	27·6	24·0	23·1 ± 2·9
Arginine	13·8	21·2	21·6	18·9 ± 2·5
Total	38·6	61·4	58·8	52·9 ± 7·2

* Averages and standard errors of the means from the 3 pools of hemolymph derived from 2 colonies of roaches.

A second unknown peak consistently occurred immediately preceding the small aspartic acid peak. Paper chromatograms of the desalted effluent showed two components, the larger of which moved in a fashion identical with methionine

sulfoxide and the smaller with methionine sulfone. The peak was at the locus of methionine sulfoxide as identified by Moore, Spackman, & Stein (1958). On the basis of its performance on paper it was assumed to consist entirely of oxidized methionine and was calculated as methionine as shown in Table 1. Methionine tends to be oxidized rather readily under column conditions unless the column is kept at a low oxidation-reduction potential with thiodiglycol. When this reducing substance was omitted the small methionine peak disappeared entirely and the methionine sulfoxide peak preceding aspartic acid was enlarged. The methionine sulfoxide peak was never suppressed by thiodiglycol (2,2′-thiodiethanol), however, indicating that the oxidized amino acid exists in roach hemolymph or arises by enzymatic or other oxidation during collecting and processing of the blood.

Cysteine cannot be determined quantitatively by the method used here. It is converted to cystine by allowing the deproteinized solution to stand at room temperature and at pH 8 for 4 hr (Stein & Moore, 1954). Even with this procedure no peak corresponding to cystine was ever observed in roach hemolymph.

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DISCUSSION

These results derived from ion-exchange columns are in accord with observations of high free amino acid concentrations in other insects. The total free amino acid concentration of the blood of the American cockroach is comparable with that of other relatively primitive insects such as the walking stick and dragonfly (Duchâteau & Florkin, 1958), but is only $\frac{1}{2}$ to $\frac{1}{3}$ those of the higher holometabolous insects such as the silkworm (G. Wyatt *et al.*, 1956) or the honeybee (Duchâteau & Florkin, 1958).

In the pooled hemolymph samples, wide variation is seen in the concentrations of certain amino acids, notably glutamic acid and proline. The most obvious explanation may be sought in dietary influences which were not controlled in the present study, although many factors, such as age, season, or time of bleeding in relation to activity cycles, could be involved. That the differences observed are not a function of source of colony is shown by the fact that the greatest fluctuation in concentration was seen in the glutamic acid of hemolymph pools from the Rutgers colony. Similar extremes of variation have been observed in other species of insects in studies on hemolymph of single specimens (G. Wyatt *et al.*, 1956) and on pooled hemolymph specimens (Duchâteau & Florkin, 1958). Total concentrations of acid and neutral amino acids and of basic amino acids were slightly lower in the St. Louis roaches, but the significance of this difference is doubtful in the absence of additional data.

These findings suggest that individual and total amino acid levels are not closely regulated in this insect. Further studies to determine the usefulness of these data for synthesizing a medium for cockroach tissue culture will be carried out.

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FREE AMINO ACIDS IN SERA OF THE MARINE INVERTEBRATES, *CANCER IRRORATUS*, *LIMULUS POLYPHEMUS* AND *HOMARUS AMERICANUS**

T. M. STEVENS, C. E. HOWARD and R. W. SCHLESINGER

Department of Microbiology, Saint Louis University School of Medicine, Saint Louis, Missouri, and
Mount Desert Island Biological Laboratory, Salisbury Cove, Maine

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Abstract—1. The free amino acids in serum of the marine arthropods *Limulus polyphemus*, *Cancer irroratus*, and *Homarus americanus* have been analyzed quantitatively by column chromatography.

2. The amino acid compositions are not remarkably specific, but the *Cancer irroratus* serum contains a strikingly large amount of taurine (17 per cent of total free amino acids measured).

3. The total free amino acid content of *Limulus polyphemus* serum is only about one-fifth that of the other two marine arthropods examined.

4. Comparative values are presented for sixteen amino acids found in the serum of these three species, in hemolymph of three insect species, and in human serum.

5. *Homarus americanus* and *Cancer irroratus* have total amino acid concentrations comparable to that of man, and less than 10 per cent of those found in insects.

INTRODUCTION

THE preceding paper (Stevens, 1961) reported results of column chromatographic analyses of the free amino acids in hemolymph of the American cockroach, *Periplaneta americana*. The primary purpose was to obtain guidance in the preparation of defined media for *in vitro* cultivation of tissues or cells of this insect in analogy to the earlier work of G. Wyatt *et al.* (1956) on the silkworm. Since success in obtaining cell proliferation depends on incorporation of 5 to 20 per cent hemolymph in the growth medium (S. Wyatt, 1956; Grace, 1958; Jones & Cunningham, 1960), the limited quantities of hemolymph which can be obtained conveniently determine in part the scale on which work of this kind can be carried out.

The amino acid analyses were extended to sera of three species of large marine arthropods because they are available in large amounts and might be suitable substitutes for cockroach hemolymph as additives to growth media. Although this possibility has not yet been explored, the free amino acid contents of the sera in themselves provide a basis for comparison with those of other animals which have been similarly analyzed. Such comparative data form part of this report.

The sera of the following three species of marine arthropods were analyzed for their free amino acid content: the horseshoe crab (*Limulus polyphemus*), the rock

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crab (*Cancer irroratus*), and the American lobster (*Homarus americanus*). Free amino acid studies on the blood of species closely related to the American lobster have previously been reported. Camien *et al.* (1951) analyzed the blood of the European lobster, *Homarus vulgaris*. Analyses by the same workers on the blood of *Astacus fluviatilis*, a freshwater crayfish, were reported by Duchâteau & Florkin (1954).

TABLE 1—FREE ACID AND NEUTRAL AMINO ACIDS OF *Limulus polyphemus*, *Cancer irroratus*, AND *Homarus americanus* (mg/100 ml)

	Limulus		*Cancer*		*Homarus*
	Pool 1	Pool 2	Pool 1	Pool 2	Pool 1
Taurine	0·17	0·12	4·20	6·15	1·90
Methionine sulfoxide	0·00	0·04	0·26	0·59	0·23
Aspartic acid	0·16	0·21	0·33	0·23	0·69
"Threonine"	0·17	0·15	1·12	0·75	0·65
"Serine"	0·62	0·66	1·99	3·48	2·38
Glutamic acid	0·33	0·26	1·44	0·59	0·16
Proline	1·50	0·83	3·36	4·16	6·90
Glycine	0·29	0·22	3·00	3·67	2·12
Alanine	0·14	0·18	1·42	1·89	2·83
Valine	0·35	0·17	1·64	1·37	0·93
Methionine	trace	trace	0·14	0·46	0·31
Isoleucine	0·12	0·10	0·78	0·91	0·69
Leucine	0·21	0·17	1·06	1·23	0·87
Tyrosine	0·06	0·08	1·48	1·53	0·96
Phenylalanine	0·04	0·09	0·56	—*	0·30
Total	4·16	3·28	22·78	27·01	21·92

* Not determined.

MATERIALS AND METHODS

Collection of blood

The three arthropods were bled through the dorsal intersegmental membrane between thorax and abdomen. The average horseshoe crab yielded over 100 ml of blood; the lobster, 30 to 40 ml; and the rock crab, 10 to 20 ml. As rapidly as possible, the blood was collected in a beaker and stirred with glass rods to prevent clotting. It was then centrifuged at low speed to remove cells and clot remnants, and the supernatant plasma was frozen at $-20°C$.

Deproteinization of sera, resins used, and limits of reproducibility were as reported in the accompanying paper (Stevens, 1961) with the exception that 5–15 ml aliquots of serum were deproteinized and placed on the columns for each run and that 2 ml effluent fractions were collected in each instance.

RESULTS

The concentrations of acid and neutral free amino acids for the three marine arthropods are listed in Table 1. The results for *Limulus* and *Cancer* represent the

averages of aliquots from two pools of blood for each species. Each pool consisted of the blood of two to ten animals. The *Homarus* data in Table 1 represent a single chromatogram on the pooled sera of five individuals.

The most striking finding was the presence of large amounts of taurine (about 17 per cent of all free amino acids measured) in the serum of *Cancer irroratus*. The concentration of taurine in *Limulus* and *Homarus* was considerably lower.

Table 2—Free basic amino acids of *Limulus polyphemus*, *Cancer irroratus*, and *Homarus americanus* (mg/100 ml)

	Limulus		*Cancer*		*Homarus*	
	Pool 2	Pool 3	Pool 2*		Pool 2*	
Lysine	0·46	0·74	1·91	1·93	0·42	0·49
Histidine	+†	+†	0·66	0·62	0·19	0·18
Arginine	0·56	0·49	3·26	3·80	1·01	1·00
Total	1·02	1·23	5·83	6·35	1·62	1·67

* Duplicate runs on same pooled sample.
† Present but not quantitatively determined due to interference by NH_3.

Threonine and serine were consistently represented by composite peaks with a tendency to overlap. It has been shown that glutamine and asparagine are eluted from the column in the same region (Tallan, Moore, & Stein, 1954). The amount of color due to these substances is unknown, since they are not quantitatively determined by this method. Consequently, threonine and serine are enclosed in quotation marks in the first column.

Table 2 shows the concentrations found for the basic free amino acids in pooled samples of blood.

DISCUSSION

A comparison of serum amino acid contents of several arthropod species and of man is presented in Table 3. Only sixteen amino acids are shown because they were determined by all the investigators whose data were used in this compilation. For easier comparison, the data are expressed in terms of percentages based on the total of these sixteen free amino acids. At the bottom of Table 3 total concentrations in mg/100 ml are also included.

The individual amino acids show considerable variation in proportions from one species to another. Glycine or proline or both are in high proportional concentration in most of these species; aspartic acid and phenylalanine are uniformly low. The data do not support any further generalization.

The total free amino acid contents in hemolymph of the three insects, *Periplaneta*, *Carausius*, and *Bombyx*, are characteristically high. However, *Periplaneta* and *Carausius*, which belong to the primitive order Orthoptera, have relatively lower concentrations than *Bombyx mori* which represents the more specialized

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holometabolous insects. This characteristic of increasing levels of free amino acids with increasing evolutionary development was first observed by Duchâteau & Florkin (1958). An interesting parallel is suggested by the results obtained with the three marine arthropods, in that the lowest free amino acid concentration is found in the blood of the primitive xiphorsuran, *Limulus polyphemus*, while the *Homarus* and *Cancer* species have concentrations four to five times as high.

TABLE 3—COMPARISON OF AVERAGE CONCENTRATIONS OF 16 FREE AMINO ACIDS IN DIFFERENT ANIMALS. (% OF TOTAL OF 16 AMINO ACIDS)

	Marine arthropods			Insects			
	*Limulus polyphemus**	*Homarus americanus**	*Cancer irroratus**	*Periplaneta americana*† adult	*Carausius morosus*‡ adult	*Bombyx mori*§ larva	Man‖
Aspartic acid	4·0	3·2	1·1	0·8	1·1	1·3	0·1
Threonine	3·4	3·0	3·6	2·8	12·2	4·4	5·9
Serine	13·6	10·9	10·5	5·2	—	11·8	4·7
Glutamic acid	6·4	0·8	3·9	8·8	2·6	1·7	3·0
Proline	24·7	31·8	14·4	15·7	4·6	4·6	10·0
Glycine	5·5	9·8	12·8	19·5	6·7	9·2	6·5
Alanine	3·4	13·0	6·3	2·7	16·5	4·3	14·4
Valine	5·5	4·3	5·8	4·0	7·2	3·9	12·2
Methionine	trace	2·5¶	2·8¶	4·9¶	5·5	2·6	1·6
Isoleucine	2·3	3·2	3·3	2·1	4·0	4·4	3·8
Leucine	4·1	4·0	4·4	2·5	4·0		7·2
Tyrosine	1·5	4·4	5·8	9·3	2·5	4·5	4·3
Phenylalanine	1·5	1·4	2·1	2·3	2·6	1·1	3·5
Lysine	12·8	2·1	7·3	4·0	6·0	17·2	11·5
Histidine	+**	0·9	2·4	8·5	18·7	25·4	4·9
Arginine	11·3	4·7	13·5	6·9	5·8	3·6	6·4
Total %	100·0	100·0	100·0	100·0	100·0	100·0	100·0
Total Mg/100 ml	4·7	21·7	26·1	271·3	347·3	816	23·64

* See Tables 1 and 2.
† See Tables 1 and 2 of accompanying paper (Stevens, 1961).
‡ Duchâteau & Florkin, 1958. Microbiological assays of dialysate of pooled hemolymph.
§ G. Wyatt *et al.*, 1956. Averages of data from paper chromatograms of fourteen individuals.
‖ Stein & Moore, 1954.
¶ Includes methionine sulfoxide. See accompanying paper (Stevens, 1961).
** See Table 2.

The data on free amino acid levels in human serum are included for reference. They are indicative of levels generally found in higher animals, cf. rooster, rat, cat, and dog (Duchâteau & Florkin, 1958). The rock crab and the American lobster have total free amino acid concentrations comparable to those of man and higher animals but less than 10 per cent of those found in insects. This confirms a similar comparison by Florkin (1956) involving two other crustaceans, *Homarus vulgaris* and *Astacus fluviatilis*.

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SHORT COMMUNICATION

HISTAMINE CONTENT OF TISSUES FROM THE CRAB *CARCINUS MAENAS*

G. A. KERKUT and M. A. PRICE

Department of Physiology and Biochemistry, The University of Southampton

(*Received* 2 *June* 1961)

Abstract—The tissues of the crab have histamine contents varying from 1212 μg/g in the case of the heart to 1·3 μg/g for the gill. The tissues high in histamine are heart, pericardial organs, and pericardium. The other tissues have a low histamine content (1·3–21 μg/g).

HISTAMINE has been described to be present in the tissues of various invertebrates (Ungar, Ungar & Parrot, 1937; Parrot & Reuse, 1954) and techniques for its separation and estimation from homogenates are readily available.

During the course of experiments on the crab heart, we found that there was a high concentration of histamine. We therefore made a survey of the histamine content of the various crab tissues to see if the levels were significant.

Carcinus maenas were freshly obtained from the Solent and their tissues removed for analysis. In the case of minute organs such as the pericardial organs, the organs from at least 50 crabs were used for each estimate. This provided about 17–25 mg of tissues, enough for many multiple chromatogram runs. At least five separate assays were carried out for each tissue, and in most cases the number of experiments was more. Fatty tissues such as the hepatopancreas were ether extracted to prevent the fat from interfering with the running of the chromatograms. The tissues were homogenized, the protein precipitated, the solution centrifuged and the clear supernatant removed and evaporated to small volume. Descending and two-way chromatograms were developed in solvents such as *n* propanol-ammonia; *n* butanol-water; methanol-tert-butanol-water; 70 per cent propanol; 60 per cent acetone; butanol HCl. Histamine base and histamine hydrochloride were used as standards and markers. The histamine was visualized by spraying with Pauly's reagent—the histamine coming up a rose red, or with para-nitroaniline (orange red). The quantities were determined photometrically or from direct standards. Pauly's reagent under controlled conditions with histamine gave colours that obeyed Lambert and Beer's laws between 1 μg and 20 μg of histamine.

The results are shown in Table 1. It will be seen that there are two classes of crab tissues. The heart, pericardial organs, and the ventral pericardium have a high histamine content (870–1212 μg/g). The remaining tissues have a low content (1·3–21 μg/g).

The heart has a higher histamine content than does skeletal muscle. Similarly the pericardial organs have a higher content than nervous tissue such as the stellate ganglia. There is, however, one problem concerning the pericardial organs. It is not clear the extent to which their high histamine content depends on the pericardial connective tissue surrounding them. Our view is that they have a high histamine content on their own accord. Analysis of gross sections indicated that there was no regional localization of histamine in the heart.

TABLE 1

Tissue	Histamine concentration
Heart	1212 μg/g
Pericardial organs	909 μg/g
Ventral pericardium	877 μg/g
Stellate ganglia	21 μg/g
Blood	13·9 μg/ml
Testes	12 μg/g
Hepatopancreas	3·6 μg/g
Muscle	2·4 μg/g
Ovary	1·8 μg/g
Gill	1·3 μg/g

The high concentrations of histamine in the crab heart compare favourably with the highest histamine concentrations recorded from other living sources. The high concentration of 1290 μg/g has been found in the mast cell tumours of the dog, and human mast cell tumours have 950 μg/g (Riley & West, 1953). In the stinging nettle, Emmelin & Feldberg (1947) reported a histamine content of 1000 μg/ml. Otherwise the levels of histamine are relatively low; nervous tissue such as ox splenic nerve had 98 μg/g, whilst ox brain had 0·1–1·0 μg/g (von Euler, 1956). Skin from cat's ears had 95–120 μg/g; guinea-pig's abdominal skin—3·2 μg/g; rat liver—0·3 μg/g; ox aorta—10 μg/g (Feldberg, 1956).

The precise function of histamine is not clear. We had thought it might play a role in the maintenance and regulation of the heart beat. However, the heart does not appear to be very sensitive to histamine; a concentration of 10^{-3} bringing about an acceleration of the beat. There is evidence that some of the histamine in the crab heart is present as acetyl histamine, and it is possible that this or some other histamine complex may be of physiological importance.

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BOOK REVIEW

Hypothermia and the Effects of Cold. British Medical Bulletin Vol. 17. No. 1. Pub. Medical Department, The British Council, 65, Davies Street, London, W.2, 1961. 78 pp.

THE dramatic clinical advances of the last decade which stem from the use of hypothermia have stimulated the production of a number of recent publications in this field. This symposium, under the Chairmanship of Dr. A. S. Parkes, F.R.S., is a notable addition and consists of contributions from seventeen workers actively engaged in the field. It contains papers on the physiological and biochemical changes resulting from cold exposure in a wide range of non-anaesthetized animals, and includes some consideration of hibernation in animals, including birds. The functional responses induced by long-term exposure to environments at constant temperatures, below the normal animal level, are also considered.

The major part of the symposium is devoted to consideration of hypothermia—a state in which the body temperature is lowered substantially below normal. The earlier and current clinical techniques of moderate and profound hyperthermia are described and experimental work, particularly on dogs, is also reported for deep hypothermia. There are reviews of some of our current knowledge on the circulation, metabolism, the hormones and central nervous system in the hypothermic mammal. Finally there are two papers of particular interest in the field of application of hypothermia, concerning the effect of temperature on the response to drugs and the sensitivity of hypothermic mammals to X-irradiation.

To cover fully so wide a range of subjects in this rapidly developing field, in less than 100 pages, is an impossible task and probably the major limitation of this otherwise excellent brief survey. Certain papers are very specialized although their title and introduction suggest otherwise. For example, the paper on the cold resistance of poikilothermic animals is almost wholly devoted to work on insects, making little reference to that in other animal groups. Similarly, certain of the review papers lack adequate current references to provide a comprehensive summary of recent investigations on the specified subjects for other than those with a general interest in the subject.

The application of hypothermia by the clinicians has far outpaced our fundamental knowledge of the processes and factors involved and this symposium is to be commended in that the introduction and content make a significant contribution in emphasizing the discrepancy. For example, all too little is known concerning the specific and general endocrine responses of the homoiotherm to either acute or chronic lowered environmental temperature and only recently has it been established that qualitative as well as quantitative physiological and biochemical metabolic changes are induced by alteration in body temperature. Further, the species specificity, the effect of age, and the variable result of different types of anaesthesia which characterize the individual animal's response to cold, all complicate the information available to the clinician. Much more animal and clinical work is required before such simple phenomena of hypothermia as the variation in electrolyte levels and buffering capacity of the blood, and the tendency of the cardiac muscle to fibrillate, and general tissue metabolism, are adequately understood at lowered body temperature.

The experimental worker and clinician are provided in these collective papers with a ready introduction to current knowledge and practice in their colleagues' field, and this points the way to a most fruitful collaborative future, and is not the least valuable feature of a most welcome addition to these excellent British Medical Bulletins.

K. A. MUNDAY